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# Thiol modulation of the thylakoid ATPase. Lack of oxidation of the enzyme in the presence of $\Delta\tilde{\mu}_{H^+}$ in vivo and a possible explanation of the physiological requirement for thiol regulation of the enzyme

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**(1) Illumination of *Dunaliella* induced an increase in the activity of $CF_1$-ATPase, and of fructose-1,6-bisphosphatase (FBPase), that could be assayed under standard conditions in subsequently lysed cells. The light-induced, but not the light-independent, activities could be prevented by inclusion of methyl viologen in the preillumination medium. This effect was concluded to be due to the prevention of reduction of ferredoxin by methyl viologen. (2) Markedly higher concentrations of added methyl viologen were required to prevent induction of ATPase than FBPase. Addition of 1 mM methyl viologen to the preillumination stage prior to illumination totally prevented the appearance of light-induced FBPase, whereas a concentration of 20 mM was required to prevent light-induction of ATPase completely. (3) Although methyl viologen added to intact algae in the light subsequent to achievement of steady-state light activation of ATPase and FBPase led to the inactivation of FBPase, light-induced ATPase was completely uninhibited. This effect indicates that whereas FBPase is subject to reversible modification by thiols in the light, thiol reduction of ATPase in vivo is irreversible in the light, and oxidation only occurs upon dissipation of $\Delta\tilde{\mu}_{H^+}$ produced by darkening. (4) Stabilisation of the thiol-reduced form of $CF_1$ by $\Delta\tilde{\mu}_{H^+}$ (Shahak, Y. (1985) J. Biol. Chem. 260, 1459–1464) accounts for the lower reducing pressure necessary to activate thiol-dependent ATPase, compared to FBPase. Implications for the regulation of $CO_2$ fixation are discussed. (5) Complete activation of $CF_1$-ATPase activity was induced by incubation of intact algae with dithiothreitol in the dark. It is suggested that thiol-regulation of the enzyme is necessary in vivo in order to prevent activation of an ATPase activity which would otherwise inevitably result from autocatalytic generation of $\Delta\tilde{\mu}_{H^+}$ in the dark.**

## Introduction

Several enzymes involved in photosynthesis are subject to light-dark regulation, such that the activity which is extractable from photosynthetic systems increases markedly in the light and decreases again on darkening. One of the factors thought to be responsible for such changes is reversible reduction of enzyme thiol groups [1].

Abbreviations: $CF_0$-$CF_1$/$CF_1$, chloroplast coupling factor; $P_i$, inorganic phosphate; FBP/FBPase, fructose 1,6-bisphosphate/fructose-1,6-bisphosphatase; $\Delta\tilde{\mu}_{H^+}$, difference in the electrochemical potential of protons between the stroma/external medium and the intrathylakoid space; Tricine, *N*-tris(hydroxymethyl)methylglycine; Chl, chlorophyll; PS I, Photosystem I.

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Two enzymes known to be activated by reduced thiols are the membrane-bound reversible protonmotive ATPase, $CF_0$-$CF_1$ (EC 3.6.1.3) [2–10] and the stromal fructose-1,6-bisphosphatase (FBPase) (EC 3.1.3.11) [11–16].

The former enzyme is also subject to kinetic regulation by $\Delta\tilde{\mu}_{H^+}$, which can be regarded as a substrate of the enzyme [3,17,18]. Catalysis is only observed above a threshold-'activating' value of $\Delta\tilde{\mu}_{H^+}$ [17,18]. For the oxidised enzyme, this value is higher than that required to support ATP synthesis thermodynamically, and so ATP synthesis is always observed upon $\Delta\tilde{\mu}_{H^+}$-mediated activation of this form of the enzyme. However, high rates of ATP hydrolysis can be observed under partially uncoupled conditions in the dark, if broken chloroplasts have been preilluminated with dithiols [2–3,6,10]. Since, under these conditions, the enzyme is active at $\Delta\tilde{\mu}_{H^+}$ values thermodynamically low enough to allow ATP hydrolysis, it is thought that thiol reduction lowers the magnitude of $\Delta\tilde{\mu}_{H^+}$ required to activate the enzyme [19–21]. Exactly why the enzyme should require regulation by thiols in addition to $\Delta\tilde{\mu}_{H^+}$ is not clear, although it has been shown that reduction increases the rate of ATP synthesis at suboptimal $\Delta\tilde{\mu}_{H^+}$ values, presumably due to an increase in the fraction of active enzymes [19–21].

Activation of $CF_0$-$CF_1$ by $\Delta\tilde{\mu}_{H^+}$ involves a conformational change that exposes a disulphide bridge which is normally buried in the inactive enzyme [3]. Thiol reduction occurs when this disulphide bridge is reduced by dithiols such as dithiothreitol [10]. A recent model [6] suggests that, following reduction, a further conformational change occurs causing the thiol groups to become buried again, even though the enzyme remains activated. Thus, dithiothreitol reacts more readily with the activated, oxidised enzyme, but oxidants react more readily with the deactivated, reduced form.

Under some conditions of measurement, the activity of the stromal FBPase is increased markedly by thiol reduction. Reduction lowers the apparent $K_m$ for FBP and allows the enzyme to function optimally at significantly lower pH values and $Mg^{2+}$ concentrations than are necessary for optimal activity of the oxidised enzyme [11,13–14]. Since pH values and substrate concentrations which exist in the illuminated stroma may be limiting for activity of the oxidised enzyme [22–24], reduction may function to allow maximal activity in the light with oxidation ensuring low activity in the dark. Evidence has been obtained that thiol reduction of FBPase in intact chloroplasts, protoplasts, and leaves is reversible in the light [15,16]. While addition of electron acceptors which are expected to lead to oxidation of ferredoxin decrease light-induced FBPase activity, inhibition of pathways of electron transport which are expected to compete with the enzyme for electrons from ferredoxin increases the activity under conditions where activation of the enzyme is suboptimal [15,16].

Thiol reduction is probably mediated in vivo by the stromal protein thioredoxin, which is reduced in the light by ferredoxin [12,25]. As well as functioning as an 'on–off' switch, the thioredoxin system could act to kinetically regulate enzyme activities, changing activities in accordance with changes in the rate of $CO_2$ fixation which are produced, for example, by changes in incident light intensity.

It was reported in a previous paper [9] that the unicellular alga, *Dunaliella,* represented a system in which enzyme activities could be measured almost immediately upon release from the in vivo environment. This is possible due to the lack of a rigid cell wall in this organism [26]. Results presented here show that this characteristic also allows easy manipulation of stromal conditions in vivo, enabling important information regarding enzyme regulation to be obtained. Results obtained bear on the flexibility of the kinetic control of ATPase by $\Delta\tilde{\mu}_{H^+}$ and thiols in vivo and also suggest one reason why the enzyme is subject to thiol regulation.

## Materials and Methods

*Dunaliella tertiolecta* was grown and harvested as previously described [9]. $CO_2$-dependent $O_2$ evolution was assayed in a conventional oxygen electrode (Rank Bros., Botisham, Cambridge) at 25 °C in 0.5 M NaCl supplemented with 10 mM $NaHCO_3$.

For enzyme assay, intact cells were preincubated in an oxygen electrode chamber at 25 °C

at a concentration of 150 μg chlorophyll/ml in a medium comprising 0.33 M sorbitol, 30 mM Tricine-NaOH (pH 8.0), 10 mM $NaHCO_3$. Methyl viologen and dithiothreitol were added as indicated. 100 μl aliquots were withdrawn periodically as indicated and introduced into the relevant hypotonic assay medium (25 °C). Spontaneous slow cell lysis was observed to occur provided the osmotic strength of the medium was below 100 mM total solutes. In order to ensure rapid lysis, however, the resulting algal suspension was rapidly passed through a nylon mesh of pore size 10 μm four times. This process was complete within 15 s, subsequent to introduction of the sample into the assay medium.

$CF_1$-ATPase activity was assayed in a medium containing 30 mM Tricine-NaOH (pH 8.0), 2.5 mM ATP, 2.5 mM $MgCl_2$ and 0.5 mM $NH_4Cl$. FBPase activity was assayed in a medium containing 100 mM Tris-HCl (pH 7.9), 1 mM FBP, 2 mM $MgCl_2$ and 1 mM EDTA. Both enzymes were assayed by release of $P_i$ from their respective substrates; the reaction was stopped by addition of 5% trichloroacetic acid and $P_i$ was estimated colorimetrically by measuring the absorbance of the reduced phosphomolybdate complex at 800 nm. Tentoxin was obtained from Sigma, Poole, Dorset, U.K. The *Dunaliella* strain was a gift from Dr. James Gilmour, Department of Microbiology, University of Sheffield, U.K.

## Results

Each experiment comparing ATPase and FBPase shown below was carried out on the same preincubated batch of algae. Thus, algae incubated under particular conditions were sampled, lysed, and introduced into media containing FBP or media containing ATP.

A significant ATPase activity was observed upon lysis of dark-adapted algae. It was reported previously [9], that light-induced, but not light-independent ATPase, produced by lysis of *Dunaliella* was completely inhibited by inclusion of the $CF_1$ inhibitor, tentoxin [27], in the assay medium. In order to check that the ATPase activities shown here were attributable to $CF_1$, a control, in which the assay medium contained 48 μM tentoxin, was performed. This was the case for all curves depicting ATPase activity; in no case was significant light- or dithiothreitol-induced ATPase measurable in the presence of tentoxin. Light-induced and dithiothreitol-induced rates of FBP hydrolysis were typically about 70–100 μmol per mg Chl per h. Under the conditions of assay used here, the FBPase activity extractable from intact algae which was independent of light or dithiothreitol was very low, usually less than 10 μmol per mg Chl per h.

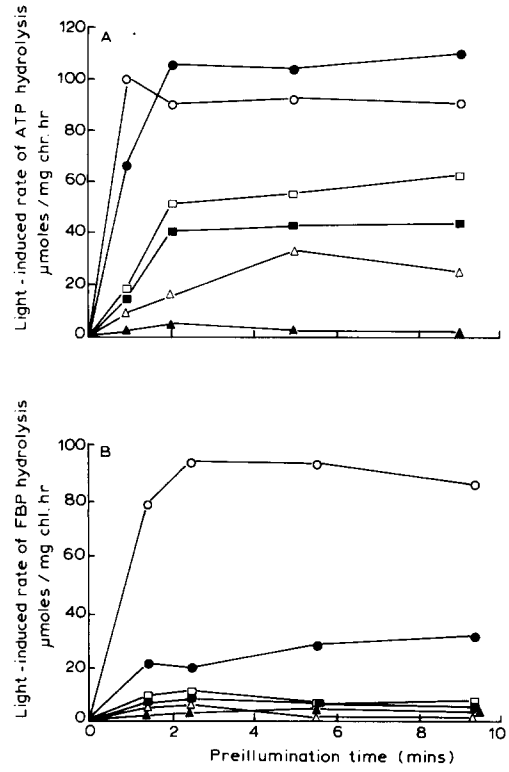


Fig. 1. Effect of methyl viologen added to intact algae in the preincubation stage 5 min prior to illumination upon light-induced ATPase (A) and FBPase activities (B). Methyl viologen concentrations: ○, control; ●, 0.5 mM; □, 1 mM; ■, 2 mM; △, 5 mM; ▲, 20 mM. Light intensity was 600 $W \cdot m^{-2}$.

Fig. 1 demonstrates that addition of methyl viologen to the preincubation medium 5 min prior to illumination inhibits both ATPase and FBPase activity observed in subsequently illuminated, then lysed, algae. The prevention of light induction of ATPase and FBPase did not result from direct inhibitory effects of methyl viologen, since inclusion of 10 mM methyl viologen in the assay media only, did not inhibit the light-induced form of either enzyme (not shown).

Table I shows evidence that methyl viologen exerted its effect by acting as an electron acceptor, and thus competing with ferredoxin for electrons from PS I. Illumination of intact algae in the absence of methyl viologen led to $CO_2$-dependent $O_2$ evolution at a rate of 166 $\mu$mol per mg Chl per h. Addition of low concentrations of methyl viologen (of the same order as those required to totally prevent light-induced FBPase) markedly inhibited the steady-state rate of $O_2$ evolution, while addition of higher concentrations caused net $O_2$ consumption, which results from oxidation of methyl viologen reduced by PS I [28]. The gradual change from net light-induced $O_2$ evolution to net light-induced consumption induced by adding increased methyl viologen concentrations presumably reflects both increasing reduction of methyl viologen and decreasing reduction of 3-phosphoglycerate, due to a dwindling NADPH supply.

A comparison of Fig. 1A and B reveals that the concentrations of methyl viologen required to prevent light-induced ATPase are considerably greater than those required to prevent light-induced FBPase. This difference is made clear by plotting the steady-state light-induced enzyme activities against the concentration of added methyl viologen (expressed logarithmically, Fig. 2). Whereas induction of FBPase activity, assayed under standard conditions, was completely prevented by the presence of 1 mM methyl viologen in the preillumination stage, a concentration of 20 mM was required to prevent ATPase totally.

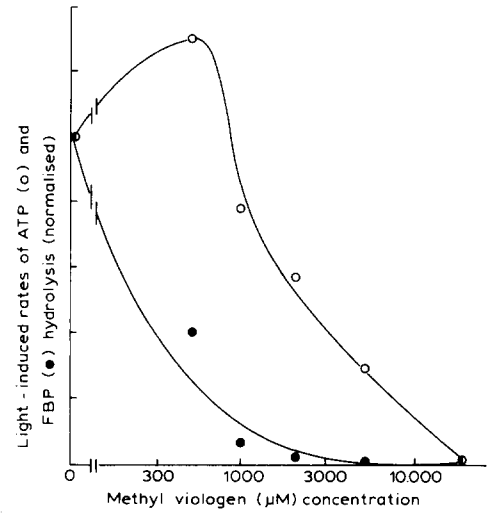


Fig. 2. Light-induced activities achieved after 9 min (ATPase) and 9.5 min (FBPase) illumination of intact algae at 600 $W \cdot m^{-2}$ plotted against the methyl viologen concentration (expressed logarithmically) in the preincubation medium. Data are normalised as a percentage of the rates achieved after the same illumination time in the absence of methyl viologen. These were 86 (ATPase) and 87 (FBPase) $\mu$mol per mg Chl per h.

TABLE I

EFFECT OF METHYL VIOLOGEN UPON PHOTOSYNTHETIC $O_2$ EVOLUTION BY INTACT *DUNALIELLA* CELLS

Methyl viologen at the indicated concentration was added to a suspension of algae (20 $\mu$g chlorophyll per ml) 5 min before turning on the light. The rate of $O_2$ evolution was measured in a medium containing 0.5 M NaCl and 10 mM $NaHCO_3$, upon achievement of the steady-state rate in the light. Positive rates, $O_2$ evolution; negative rates, $O_2$ consumption. Light intensity was 600 $W \cdot m^{-2}$.

| Methyl viologen concentration (mM) | Rate of $CO_2$-dependent $O_2$ evolution ($\mu$mol per mg Chl per h) | |
|---|---|---|
| | rate in the dark | rate in the light |
| 0 | −32 | +166 |
| 1 | −32 | +136 |
| 2 | −36 | +16 |
| 5 | −40 | −60 |
| 20 | −52 | −120 |
| 50 | −62 | −150 |

Table II demonstrates that addition of methyl viologen concentrations as high as 20 mM to algae after 6.5 min light did not inhibit light-induced ATPase activity. Contrarily, a small increase in the rate of light-induced ATP hydrolysis was produced by addition of 5 or 20 mM methyl viologen. In other experiments (not shown), addition of methyl viologen concentrations as high as 100 mM to the preillumination stage did not inhibit ATPase activity, if added once steady-state rates had been achieved. Thus, concentrations of methyl viologen which, if added to intact algae in the dark prior to illumination totally prevent light-induced ATPase (Fig. 1), fail to inhibit the enzyme if added subsequent to light activation. In contrast, Table II shows that addition of 2, 5 or 20 mM methyl viologen to intact algae under continuous il-

lumination leads to complete inhibition of light-activated FBPase activity.

It was previously reported that incubation of intact algae with dithiothreitol in the dark allowed measurement of an FBPase activity almost as high as the light-induced activity upon subsequent lysis of the algae [9]. As shown in Fig. 3B, maximal induction of the activity requires 50 mM dithiothreitol. Incubation of algae in 20 mM dithiothreitol induced a low activity, whereas 5 mM dithiothreitol had no effect. Fig. 3A demonstrates that high rates of ATP hydrolysis can also be induced by incubation of intact cells with dithiothreitol in the dark. The ATPase activity was attributable to $CF_1$, as shown by its sensitivity to tentoxin. In contrast to the FBPase activity, significant ATPase could be induced by incubation of algae in 5 mM dithiothreitol, and in the presence of 20 or 50 mM dithiothreitol the inducible activity was as high as the maximum activity inducible by illumination (Fig. 3A).

The experiments of Fig. 3 were all carried out at a light intensity lower than 0.03 $W \cdot m^{-2}$. Even so, inclusion of 50 mM $NH_4Cl$ in the preincubation medium considerably lowered the ATPase activity inducible by dithiothreitol in the dark (not shown). This experiment has to be interpreted with caution, because the carry over of uncoupler from the preincubation to the assay medium was

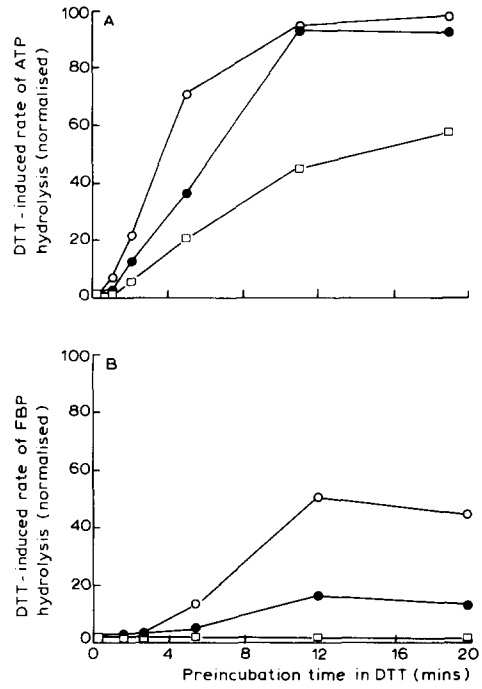


Fig. 3. Effect of incubation of intact algae in 5 mM (□), 20 mM (●), and 50 mM dithiothreitol (○) upon ATPase (A) and FBPase (B) activities measured in subsequently lysed algae. Results are normalised as a percentage of the rates produced by illumination of algae (5 min, 600 $W \cdot m^{-2}$) following 20 min in dithiothreitol. These were between 99 and 147 μmol per mg Chl per h (ATPase) and between 106 and 137 μmol per mg Chl per h (FBPase).

TABLE II

EFFECT OF METHYL VIOLOGEN ADDED TO INTACT ALGAE IN THE LIGHT UPON LIGHT-INDUCED ATPase AND FBPase

Intact cells of *Dunaliella* were illuminated at 600 $W \cdot m^{-2}$ to induce both ATPase and FBPase activities fully. Subsequent to removal of samples for assay (after 5 min light for ATPase and 5.5 min light for FBPase), methyl viologen was added to the indicated concentration (after 6.5 min light). Illumination was continued and further samples were removed at the times indicated for assay of enzyme activities.

| | Illumination time (min) | Methyl viologen concentration (mM) | | | |
|---|---|---|---|---|---|
| | | 0 | 2 | 5 | 20 |
| Light-induced ATPase | 5 | 153 | 170 | 166 | 165 |
| (μmol per mg Chl per h) | 14.5 (+MV) | 177 | 166 | 213 | 200 |
| Light-induced FBPase | 5.5 | 136 | 158 | 137 | 132 |
| (μmol per mg Chl per h) | 15 (+MV) | 123 | 19 | 15 | 16 |

shown partly to cause inhibition of dithiothreitol-induced ATPase activity (results not shown). However, the latter effect was measured in controls to be 35% of the total inhibition of dithiothreitol-induced ATPase by the presence of uncoupler in the preincubation medium, indicating that the activation of ATPase by dithiothreitol in intact cells in the dark is uncoupler-sensitive and, therefore, depends on the maintenance of a ΔpH across the thylakoid membranes.

## Discussion

In the experiments of Figs. 1 and 2 and Table II, algae were illuminated at high light intensity (600 $W \cdot m^{-2}$). Such light intensity is expected to produce maximal reduction of ferredoxin and, thus, thioredoxin. However, the redox state of

ferredoxin could be manipulated in vivo by varying the concentration of methyl viologen added to *Dunaliella* cells. Altering the redox state of ferredoxin in this way reveals that light-activated FBPase requires a much greater reducing pressure than ATPase to induce the thiol-dependent activity (Fig. 1 and 2). This is in agreement with previous data which showed that higher light intensities were necessary in order to induce FBPase than were required to activate ATPase in *Dunaliella* [9]. This effect can be explained by the model of Shahak [6]: ATPase is induced at a lower reducing pressure (lower light [9] or higher methyl viologen concentration (Fig. 1)) than is FBPase, because the thiol-reduced enzyme is effectively stabilised in the presence of $\Delta\tilde{\mu}_{H^+}$. That such stabilisation occurs in vivo is clearly illustrated by the data of Table II. Oxidant concentrations which totally prevent induction of both enzymes when added prior to illumination (Fig. 1) completely reverse light activation of FBPase if added in the light, but do not inhibit the ATPase (Table II). Indeed, at the highest concentration of added methyl viologen, a stimulation of ATPase is observed. The latter effect could be due to an increased $\Delta\tilde{\mu}_{H^+}$ under conditions where limitations due to electron acceptor availability (at high light intensity) and utilisation for ATP synthesis are likely to be lessened.

The reversibility of thiol reduction of *Dunaliella* FBPase in the light agrees with other studies of the light-activated enzyme in chloroplasts and leaves [15–16]. The kinetics of inactivation of FBPase by addition of methyl viologen in the light were linear, complete inactivation occurring within 5–8 min subsequent to addition of methyl viologen (not shown). This is somewhat slower than dark inactivation of the enzyme in *Dunaliella* [9], possibly reflecting the time taken for entry of methyl viologen into the chloroplast from the extracellular environment.

Inactivation of *Dunaliella* light-induced ribulose-5-phosphate kinase was also produced by addition of 10 mM methyl viologen to whole cells in the light (not shown), further contrasting thiol regulation of the ATPase with that of reductive pentose phosphate pathway enzymes. The irreversibility of thiol reduction of the ATPase in the light in vivo carries important implications regarding the possible significance of kinetic regulation of the ATPase to the overall process of photosynthesis. Thus, the activity of FBPase may limit, or be kinetically matched to, the rate of $CO_2$ fixation, by the redox state of thioredoxin, in a way which is not possible in the case of ATPase. Thiol modulation acts to stabilise the activated state of $CF_0$-$CF_1$ with regard to $\Delta\tilde{\mu}_{H^+}$ because the magnitude of $\Delta\tilde{\mu}_{H^+}$ required to activate the enzyme is markedly lowered by thiol reduction [19–21]. In turn, $\Delta\tilde{\mu}_{H^+}$ acts to stabilise the thiol-reduced enzyme in the light (Refs. 6 and 8, and results shown here). This complex regulation of the enzyme by $\Delta\tilde{\mu}_{H^+}$ and thiols must be seen as mechanisms which co-operate to ensure that the enzyme is always thiol-reduced and activated in the light and, hence, only subject to thermodynamic control during photosynthesis in vivo. Thus, changes in the rate of electron transport during the steady-state will only affect the thiol status of the enzyme if the light-induced $\Delta\tilde{\mu}_{H^+}$ falls below the low value which is required to activate the reduced enzyme. It therefore follows that $CO_2$ fixation is unlikely to be subject to control by the number of active ATP synthases in the steady state. Such a view does not mean that $CO_2$ fixation cannot be limited by the extent of thiol reduction of the enzyme during the induction period following a dark-light transition, where the rapidity of the initial reduction is the important consideration [9].

The activation of FBPase induced by incubation of intact cells of *Dunaliella* (Fig. 3B) with dithiothreitol agrees with results obtained using intact chloroplasts and protoplasts [29,30]. However, induction of an ATPase activity as high as that induced by light by incubation of intact algae with dithiothreitol in the dark is, at first sight, not in agreement with results obtained using broken chloroplasts [2]. Previously obtained data suggest that only the $\Delta\tilde{\mu}_{H^+}$-activated form of the enzyme can undergo significant rates of reduction [2,3,10]. Such a requirement of *Dunaliella* dithiothreitol-induced $CF_1$-ATPase for $\Delta\tilde{\mu}_{H^+}$ activation is supported by the inhibition of the activity by the inclusion of 50 mM $NH_4Cl$ during the dark incubation with dithiothreitol (results not shown).

With the above factors in mind, an explanation of the dithiothreitol-induced ATPase activity re-

quires either the existence of a significant $\Delta\tilde{\mu}_{H^+}$ in *Dunaliella* in the dark or the ability of the thiol-reduced enzyme to autocatalytically activate, by progressive generation of $\Delta\tilde{\mu}_{H^+}$, until the enzyme activity is completely induced. The latter explanation is possible because under very reducing conditions (such as would be produced by incubation with 50 mM dithiothreitol), any enzymes which become $\Delta\tilde{\mu}_{H^+}$-activated (even for a short time) will become thiol-reduced. This, possibly small, proportion of enzymes are then stabilised against deactivation, even if $\Delta\tilde{\mu}_{H^+}$ is low, since the thiol-reduced enzyme requires only a small $\Delta\tilde{\mu}_{H^+}$ for activation [19–21]. Consequent catalysis of ATP hydrolysis will increase the in vivo $\Delta\tilde{\mu}_{H^+}$ until all enzymes are both activated by $\Delta\tilde{\mu}_{H^+}$ and thiol-reduced. Important in such an explanation is the existence of significant ATP concentrations in the darkened stroma. Measured values suggest that such a situation exists, and probably results from the exchange of cytosolic and stromal triose phosphate and 3-phosphoglycerate mediated by the phosphate translocator [31,32]. The time taken for induction of ATPase by 50 mM dithiothreitol (Fig. 3A) may partly reflect the time required for generation of a $\Delta\tilde{\mu}_{H^+}$ large enough to completely activate ATPase activity. Clearly, the existence of a small $\Delta\tilde{\mu}_{H^+}$ in the dark in vivo, prior to the addition of dithiothreitol, would facilitate complete activation of the ATPase, and would mean that autocatalysis would need to occur only to a limited extent. The failure to observe complete activation of ATPase by incubation of thylakoids with dithiothreitol in the dark [2] might be due to the lack of ATP during the preincubation. Alternatively, it may reflect the lack of a $\Delta\tilde{\mu}_{H^+}$ of small magnitude which may exist in the in vivo system in dark, and which may act as a 'primer' for the autocatalytic activation of the ATPase.

Finally, the data provide an explanation of the need for both a thiol- and a $\Delta\tilde{\mu}_{H^+}$-dependent regulation of the chloroplast ATPase in vivo. Clearly, regulation of the enzyme in vivo in the dark by dissipation of $\Delta\tilde{\mu}_{H^+}$ alone would not be possible if the enzyme can simply activate itself by a progressive generation of $\Delta\tilde{\mu}_{H^+}$ through ATP hydrolysis. Only an enzyme which requires thiol reduction as well as $\Delta\tilde{\mu}_{H^+}$ activation in order to exhibit high ATPase activity can be efficiently 'switched off' by oxidation of the enzyme in the dark.

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