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Control of CO_2 fixation during the induction period. The role of thiol-mediated enzyme activation in the alga, *Dunaliella*

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(1) Illumination of the unicellular green alga, *Dunaliella*, produced a 2–3-fold enhancement of ATPase activity in subsequently lysed algae. Using the inhibitor, tentoxin, it was shown that this light-induced activity, but not the light-independent activity, was attributable to the chloroplast coupling factor, CF_1. (1) A 4–5-fold increase in fructose-1,6-bisphosphatase activity was measured in *Dunaliella* lysed subsequent to illumination. (3) Experiments with methyl viologen demonstrated that both light-induced CF_1-ATPase and fructose-1,6-bisphosphatase activities were due to thiol-modulation of the enzymes by the algal thioredoxin system. (4) The light-induced increase in fructose-1,6-bisphosphatase activity could be simulated by incubation of intact algae in the dark with dithiothreitol. This thiol-induced increase in enzyme activity was accompanied by a decrease in the induction period of CO_2-dependent O_2 evolution upon subsequent measurement. (5) The kinetics of induction of both enzyme activities were very similar to the kinetics of induction of CO_2-dependent O_2 evolution in *Dunaliella*. As the light intensity was increased to 180 $W \cdot m^2$ the steady-state enzyme activities increased in parallel with the rate of CO_2-dependent O_2 evolution. (6) The results are consistent with the imposition of a kinetic restraint on CO_2 fixation by the extent of enzyme activation under certain conditions in *Dunaliella*.

Introduction

Illumination of isolated intact chloroplasts, protoplasts or whole leaves from higher plants leads to fixation of CO_2 at a rate dictated by the prevailing environmental conditions. However, there is a lag or induction period, typically of 2–5 min, before the steady-state rate is reached. This induction period has been explained in terms of substrate- or enzyme-linked limitations during the first few minutes of illumination [1,2]. However, it is evident that there will be subtle interplay between these two factors via the phenomena of metabolite effectors of enzymes (for example, see Ref. 3).

Both the concentration of substrates and the activity of several stromal enzymes are known to increase over the first few minutes of illumination [2,4]. The increase in the activity of several stromal enzymes has been proposed to result from thiol-reduction by thioredoxin, a physiological reductant present in the stroma (for a review see Ref. 5). The thioredoxin system could represent a

Abbreviations: CF_0CF_1/CF_1, chloroplast coupling factor; P_i, inorganic phosphate; $\Delta\tilde{\mu}_{H^+}$, difference in the electrochemical potential of protons between the stroma/external medium and the intrathylakoid space; Tricine, (*N*-tris(hydroxymethyl)methylglycine).

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means of switching enzymes on in the light and off in the dark in order to prevent the operation of so-called 'futile cycles', which could occur if two antagonistic enzymes were simultaneously active. In addition, thioredoxin may function to regulate enzyme activity kinetically and thus match rates of catalysis to the rate of the overall process of CO_2 fixation. This paper presents results of experiments designed to investigate the kinetics of activation of two photosynthetic enzymes in the unicellular green alga *Dunaliella tertiolecta* as a means of assessing the contribution of enzymic limitation of the reductive pentose phosphate pathway to the lag period.

The first enzyme studied, the membrane-bound protonmotive ATPase of thylakoids, CF_0-CF_1 (EC 3.6.1.3), is known to be activated by the trans-thylakoid $\Delta\tilde{\mu}_{H^+}$ which is formed in the light [6–12]. The rate of ATP synthesis by CF_1 is thought to be dependent on the number of active enzymes and the magnitude of $\Delta\tilde{\mu}_{H^+}$, which is also a substrate of the enzyme [7]. Thiol-reduction, possibly of a disulphide bridge of the gamma subunit of CF_1, has been proposed to reduce the magnitude of $\Delta\tilde{\mu}_{H^+}$ required for activation and thereby increase the rate of ATP synthesis at a given $\Delta\tilde{\mu}_{H^+}$ [8,9]. Thus, thiol-modulation of CF_1 may play an important role in allowing high rates of photophosphorylation to be maintained at a $\Delta\tilde{\mu}_{H^+}$ which is limiting for activation of the oxidised enzyme.

The reverse reaction, ATP hydrolysis, is not observed in the light as the light-induced $\Delta\tilde{\mu}_{H^+}$ thermodynamically poises the reaction in the direction of ATP synthesis. CF_1 isolated from dark adapted leaves is also incapable of ATP hydrolysis in the dark, since, although the reaction is thermodynamically feasible, the enzyme is inactive. However, CF_1 from isolated thylakoids preilluminated in the presence of thiols is capable of high rates of ATP hydrolysis under partially uncoupling conditions in the dark [10–12]. This has been rationalised in terms of a thiol-mediated reduction in $\Delta\tilde{\mu}_{H^+}$ required for CF_1 activation during the preillumination period [8,9]. Thus, the enzyme is active at a $\Delta\tilde{\mu}_{H^+}$ which is thermodynamically small enough to allow ATP hydrolysis. Under the partially uncoupling conditions of the assay medium, only thiol-modulated enzymes are able to hydrolyze ATP, and thus rates of ATP hydrolysis are indicative of the degree of thiol-reduction of CF_1.

The second enzyme studied, the Mg^{2+}-dependent stromal fructose-1,6-bisphosphatase (EC 3.1.3.11), displays a marked dependence on both Mg^{2+} concentration and pH when assayed in vitro [13–15]. At a Mg^{2+} concentration of 10 mM and pH 8.8, enzymes from dark-adapted and illuminated chloroplasts show no difference in activity [13]. However, at a Mg^{2+} concentration of 5 mM and pH 8.0 (conditions broadly similar to those reported to exist in the illuminated stroma [16]) the activity of fructose-1,6-bisphosphatase isolated from dark-adapted chloroplasts is markedly lower than that from illuminated chloroplasts [17]. This light-induced increase in activity can be mimicked by the addition of exogenous thiols [1,15,18] or purified thioredoxin [14] and is thought to reflect thiol-modulation of the enzyme [14,15]. Thus, light-induced rates of fructose-1,6-bisphosphate hydrolysis at suboptimal Mg^{2+} concentration and pH values are indicative of the degree of thiol-reduction of fructose-1,6-bisphosphatase.

The marine halophyte, *Dunaliella*, lacks a rigid cell wall, and can be lysed by simple methods, enabling rapid assay of enzyme activity at different stages during the induction period. *Dunaliella* thus represents a true in vivo system which is more open to experimental manipulation than whole leaves from higher plants. Results obtained are interpreted as indicating that thiol-mediated enzyme activation may play a part in regulating CO_2 fixation during the induction period in *Dunaliella*.

Materials and Methods

Dunaliella tertiolecta (strain no. 19/6/A, originally from the Culture Centre for Algae and Protozoa) was grown at 25 °C with a 14/10 h day/night cycle in a medium of 1.5 M NaCl supplemented with inorganic nutrients (adapted from Ref. 19). Cells were harvested in early-mid log phase by centrifuging at $500 \times g$ for 3 min.

CO_2-dependent O_2 evolution was routinely assayed in a conventional oxygen electrode (Rank Bros., Botisham, Cambridge) at 25 °C in 0.5 M NaCl supplemented with 10 mM $NaHCO_3$. CO_2-dependent O_2 evolution was also assayed in the

preincubation medium used for enzyme assay and no difference in either rates or kinetics of induction was found relative to CO_2-dependent O_2 evolution in 0.5 M NaCl.

For enzyme assay, intact cells were preilluminated at 25 °C at a concentration of 150–200 μg chlorophyll/ml in an oxygen electrode chamber in a medium comprising 0.33 M sorbitol/30 mM Tricine-NaOH (pH 8.0)/20 mM NaCl/5 mM $MgCl_2$. Methyl viologen and dithiothreitol were added as indicated. 100-μl aliquots were withdrawn for enzyme assay after various periods of illumination and introduced into the relevant hypotonic assay medium (25 °C). Spontaneous, slow 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, twice (for ATPase), or four times (for fructose-1,6-bisphosphatase). The optimal number of passages through the mesh was experimentally determined and the process was complete within 15 s.

For ATPase assay the medium consisted of 30 mM Tricine-NaOH (pH 8.0)/2.5 mM ATP/2.5 mM $MgCl_2$/0.5 mM NH_4Cl. For fructose-1,6-bisphosphatase assay, the medium was 100 mM Tris-HCl (pH 7.9)/1 mM EDTA/1 mM fructose 1,6-bisphosphate/2 mM $MgCl_2$. Both enzymes were assayed by release of P_i according to the protocol described in detail in Ref. 20. The reaction was stopped by addition of 5% trichloroacetic acid and P_i was estimated colorimetrically by absorbance at 800 nm. Tentoxin was obtained from Sigma, Poole, Dorset, U.K. The *Dunaliella* strain was a kind gift from Dr. James Gilmour, Department of Microbiology, University of Sheffield, U.K.

Results

As stated in the Materials and Methods section, rates of both ATPase and fructose-1,6-bisphosphatase were determined by release of P_i from ATP and fructose-1,6-bisphosphate, respectively, by lysed algae. Whole cells such as *Dunaliella* contain measurable amounts of endogenous P_i, and this was determined by addition of trichloroacetic acid to the assay medium prior to addition of the algae. Endogenous P_i values as estimated by this control were low in comparison to barley protoplasts [20,23] and the absorbance at 800 nm was typically 10–15% of the value produced by illuminated algae in the presence of ATP or fructose 1,6-bisphosphate. All results shown, whether light-activated or total rates of P_i release, have been corrected for this small background P_i.

Fig. 1 shows the effect of preillumination on total ATPase activity observed in subsequently lysed *Dunaliella*. Preincubation time refers to the period of incubation of intact algae in the light or light and dark as indicated on the diagram. Points represent the time at which samples were taken and lysed to produce algal thylakoids, which were allowed to hydrolyze ATP in the dark. Illumination typically produced a 2–3-fold enhancement of ATPase activity which was completely inducible within 5 min. This increase in activity was reversible by darkening, total loss of light-induced activity occurring after 15 min darkness.

Fig. 2 demonstrates that the steady-state light-induced activity can be completely inhibited by the presence of tentoxin in the assay medium. Tentoxin is known to be an inhibitor of CF_1 isolated from certain species of higher plants [21,22]. Fig. 2 shows that tentoxin also inhibits the ATPase activity of CF_1 in *Dunaliella* thylakoids. However, the light-independent activity (dotted

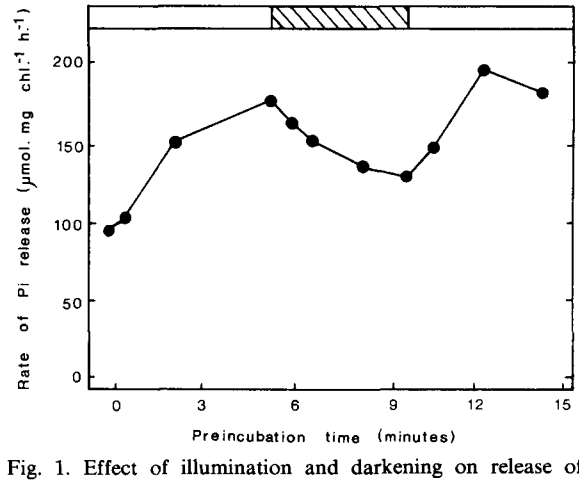

Fig. 1. Effect of illumination and darkening on release of phosphate from ATP catalysed by thylakoids from intact algae lysed subsequent to light, light + dark, or light + dark + light treatment. Clear areas, illumination (Light intensity = 68 W · m^{-2}); Hatched areas, darkness.

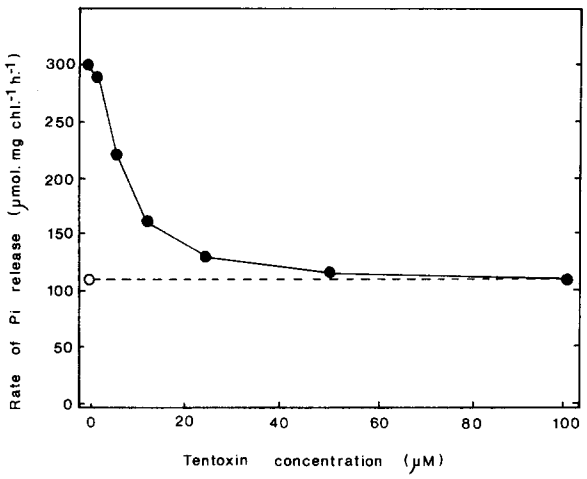

Fig. 2. Effect of tentoxin on ATPase activity of thylakoids released from *Dunaliella* subsequent to 5 min illumination at a light intensity of 105 $W \cdot m^{-2}$ (the open symbol and dotted line indicate dark-adapted algae). Tentoxin was present in the assay stage only.

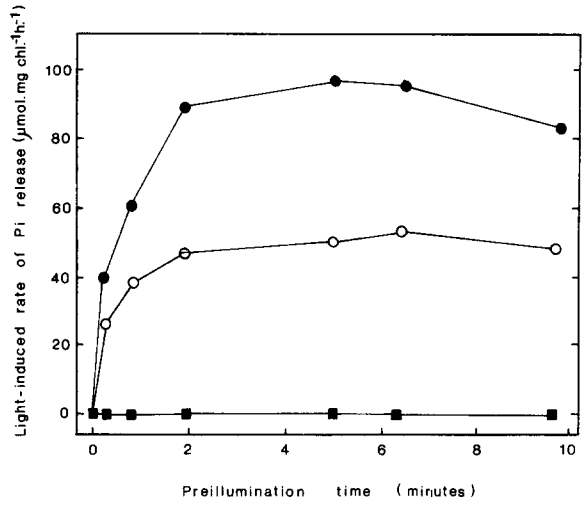

Fig. 3. Effect of addition of methyl viologen to intact algae in the preincubation stage on rates of light-induced ATP hydrolysis by subsequently lysed algae. ●, control (no methyl viologen); ○, +10 mM methyl viologen; ■, +100 mM Methyl viologen. Light intensity was 105 $W \cdot m^{-2}$.

line, Fig. 2) was unaffected by tentoxin, indicating that this activity is not attributable to CF_1, and probably results from the action of non-specific cellular phosphatases. The light–dark reversible nature of *Dunaliella* CF_1-mediated ATP hydrolysis and its inhibition by tentoxin agrees with data obtained with isolated barley protoplasts [20,23].

In an attempt to demonstrate that the light-induced CF_1-mediated ATPase activity is regulated by a reductant generated in the light by electron transport, the exogenous electron acceptor methyl viologen was added to the preincubation stage 1 min prior to illumination. Fig. 3 shows that the presence of methyl viologen in the preillumination period caused adjustment of subsequent dark period ATPase to a new, lower steady-state rate. Light-induction of ATPase was totally prevented by 100 mM methyl viologen, whereas no effect was observed on light-independent ATPase activity. A control experiment, in which the same concentrations of methyl viologen were added to intact algae in the light subsequent to the achievement of steady-state rates of ATP hydrolysis showed that methyl viologen had no effect on pre-reduced enzyme (data not shown). The prevention of light-induced ATPase by methyl viologen, which supports formation of a $\Delta\tilde{\mu}_{H^+}$ but which prevents reduction of ferredoxin, indicates that CF_1-mediated ATP hydrolysis is dependent upon a reductant generated in the light by electron flow from ferredoxin. Such a reductant is probably thioredoxin [5].

Fig. 4 shows a comparison between the kinetics of induction of the reductant-controlled, CF_1-mediated ATPase activity and those of CO_2-dependent O_2 evolution in *Dunaliella*. The figure demonstrates that increasing the light intensity up to 105 $W \cdot m^{-2}$ increased the steady-state rate of both CO_2-dependent O_2 evolution and ATPase. At low light intensities the percentage of ATPase activity induced was virtually identical to the percentage of CO_2-dependent O_2 evolution induced, both during the lag and during the steady state. However, at light intensities of 100 $W \cdot m^{-2}$ or above, although normalised steady-state rates of CO_2-dependent O_2 evolution and ATPase were virtually identical, the illumination time required to attain this rate was significantly shorter in the case of ATPase. Thus at light intensities greater than 100 $W \cdot m^{-2}$ thiol-modulation appears to be complete when CO_2 fixation is only 50–70% of the maximum rate inducible at those light intensities.

Fig. 5 shows a typical light-activation/dark-deactivation plot for fructose-1,6-bisphosphatase in *Dunaliella*. Illumination produced a 4–5-fold

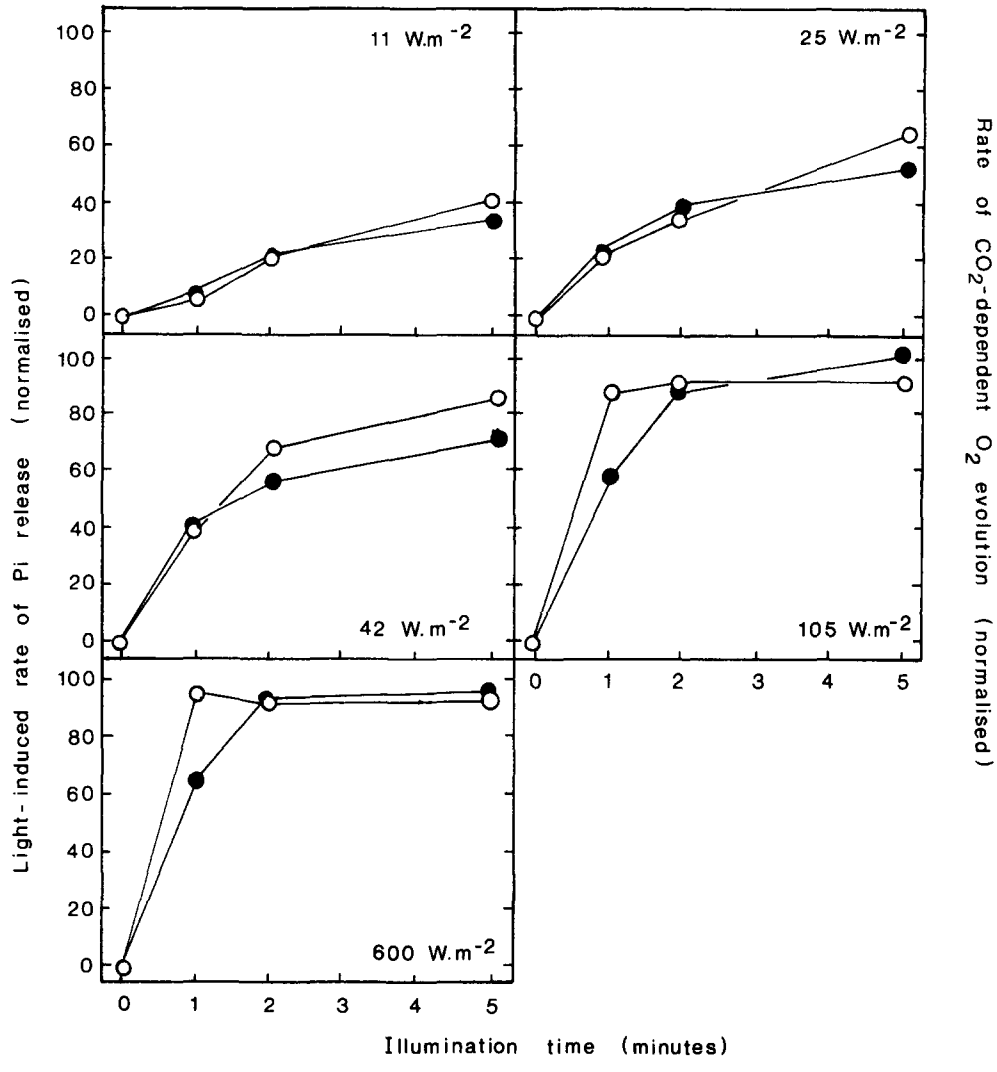

Fig. 4. Comparison of kinetics of induction of light-induced ATP hydrolysis and CO_2-dependent O_2 evolution in *Dunaliella* at five different light intensities. Results are normalised as a percentage of the high light control. ○, ATP hydrolysis; ●, CO_2-dependent O_2 evolution. Each point represents the mean of 8 data. Standard errors were typically 5–15% of the mean. Maximum rates of CO_2-dependent O_2 evolution and light-induced ATP hydrolysis at high light were typically 100–120 and 80–100 μmol/mg Chl per h, respectively.

increase in activity which was dark-reversible. The decrease in activity seen upon darkening occurred more quickly for fructose-1,6-bisphosphatase than for ATPase (cf. Fig. 5 with Fig. 1).

Fig. 6 shows results obtained by the same methods and protocol as Fig. 4 but here subsequently lysed algae were assayed for fructose-1,6-bisphosphatase activity instead of ATPase. The data again demonstrate that thiol-modulation proceeded with kinetics similar to those of the induction of CO_2-dependent O_2 evolution. However, the data contrast slightly with those obtained for ATPase in that thiol-modulation followed the kinetics of CO_2-dependent O_2 evolution more closely at high light intensity rather than low. This may partly reflect the presence of some stromal fructose-1,6-bisphosphatase in the dark, which may contribute to the rate of CO_2-dependent O_2 evolu-

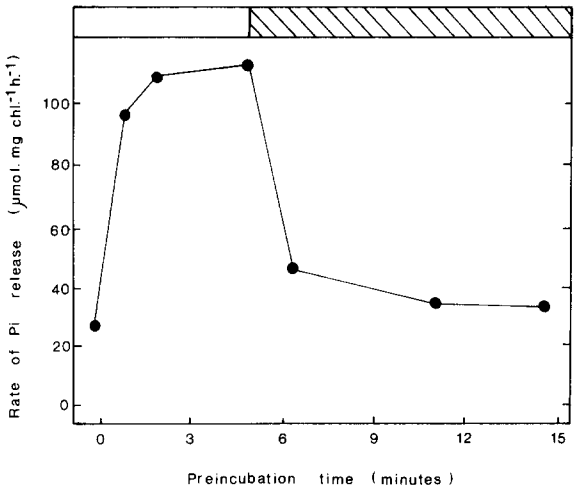

Fig. 5. Effect of illumination and darkening on release of phosphate from fructose-1,6-bisphosphate catalysed by thylakoids released from algae lysed subsequent to light or light + dark treatment. Clear areas, illumination (light intensity = 90 W·m^{-2}); Hatched areas, darkness.

tion. It is unclear as to whether this light-independent fructose-1,6-bisphosphatase activity represents stromal or cytoplasmic enzyme activity. Experiments with fructose 2,6-bisphosphate, a known inhibitor of the cytoplasmic enzyme [5], are currently in progress in order to answer this question. Experiments in which the preillumination period was carried out in the presence of methyl viologen (data not shown) indicated that the light-induced fructose-1,6-bisphosphatase activity is attributable to thiol-induced stromal fructose-1,6-bisphosphatase. Light-induced activation of fructose-1,6-bisphosphatase was completely prevented by including 10 mM methyl viologen in the preincubation medium. In addition, the steady-state light-induced fructose-1,6-bisphosphatase activity was rapidly inhibited by addition of 10 mM methyl violgen to the preincubation stage in the light.

Fig. 7 shows additional evidence for the attribution of light-induced fructose-1,6-bisphosphatase to thiol-regulated stromal fructose-1,6-bisphosphatase. Intact algae were preincubated for up to 60 min either in the light, or in the dark in the presence of exogenous dithiothreitol. As Fig. 7 shows, incubation of intact algae in the dark with 50 mM dithiothreitol for 60 min produced a fructose-1,6-bisphosphatase activity which was approx. 80% of the maximal light-induced activity. Illumination subsequent to incubation with dithiothreitol (not shown) produced a fructose-1,6-bisphosphatase activity equal to that produced by illumination alone, indicating that the light-activated and dithiothreitol-induced activities are attributable to the same enzyme.

Fig. 7 indicates that stromal enzymes of *Dunaliella* can be thiol-activated by incubation of intact cells with exogenous thiols in the dark. In order to assess the effect of pre-reduction of stromal enzymes in the dark upon the induction period of CO_2 fixation, CO_2-dependent O_2 evolution was measured by illumination of samples of algae taken immediately prior to, and subsequent to, incubation in 50 mm dithiothreitol for 60 min. The lag in CO_2-dependent O_2 evolution was calculated by linear extrapolation of steady-state rates back to the point of intercept with a linear extrapolation of the rate at the point the light was turned on. Typically, it was found that concomitant with the dithiothreitol-induced fructose-1,6-bisphosphatase activity was a dithiothreitol-induced shortening of the induction period of CO_2-dependent O_2 evolution. The extent of shortening of the lag varied from 0–50% in six experiments.

Discussion

Results presented here show that the thiol-mediated activation of both CF_1-ATPase, which has been demonstrated in isolated higher plant chloroplasts [11,12] and protoplasts [20,23], and stromal fructose-1,6-bisphosphatase, which is well documented in leaves [24], protoplasts [1,25], and chloroplasts [1,2,13,17,18,25] from higher plants, occurs in the alga *Dunaliella* and can be reproducibly measured in the dark following a period of illumination.

The fungal tetrapeptide, tentoxin, is known to inhibit membrane-bound CF_1-ATPase activity in barley protoplasts [20,23]. It is also known to inhibit ATPase activity of CF_1 isolated from some higher plants, notably lettuce, spinach and certain species of tobacco [21,22], although the extent of inhibition is greatly increased by preincubation times of 10–60 min [21,22]. However, even with preincubation times of this length, no effect of 2.4 μM tentoxin has been observed on ATPase activity of isolated radish CF_1 [21] and 6 μM tentoxin

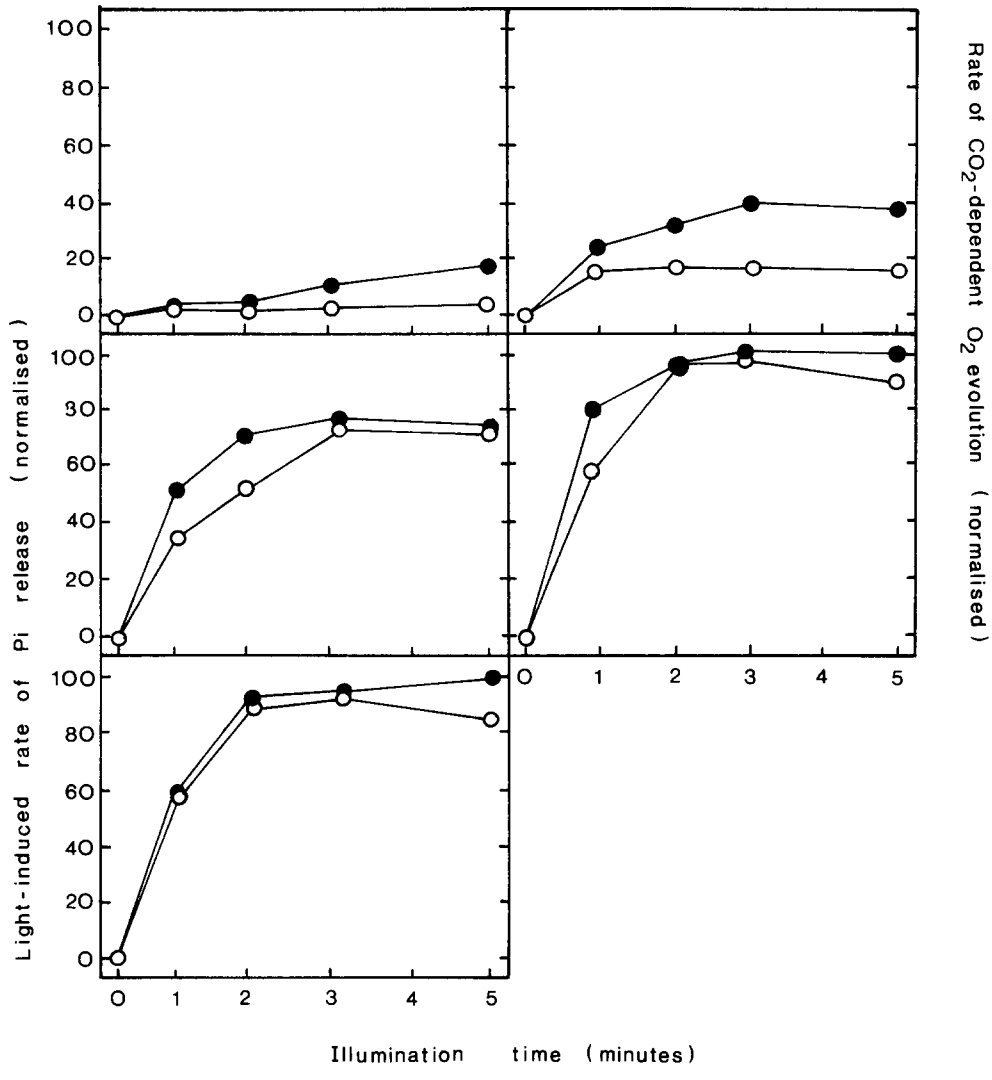

Fig. 6. Comparison of kinetics of induction of light-induced fructose-1,6-bisphosphate hydrolysis and CO_2-dependent O_2 evolution at five different light intensities. Results are normalised as a percentage of the high light control. ○, fructose-1,6-bisphosphate hydrolysis; ●, CO_2-dependent O_2 evolution. Maximum rates of CO_2-dependent O_2 evolution and light-induced fructose 1,6-bisphosphate hydrolysis at high light were 168 and 154 μmol/mg Chl per h, respectively.

only marginally inhibited (10–20%) isolated CF_1 from other, 'insensitive' species of tobacco [22]. In view of this species-specificity in higher plants, it is perhaps surprising that, even in the absence of a preincubation period (which was not possible in these experiments), 6 μM tentoxin inhibited steady-state light-induced ATPase in *Dunaliella* thylakoids by 50% (Fig. 2).

At light intensities above 100 $W \cdot m^{-2}$ (Fig. 4) *Dunaliella* ATPase activity was induced by illumination times similar to those for light-induced ATPase activity in barley protoplasts [23]. However, at lower light intensities, *Dunaliella* ATPase required up to 5 min preillumination for complete induction. In barley protoplasts, light-induced ATPase activity was saturated at light intensities of 10 $W \cdot m^{-2}$ [20], whereas Fig. 4 shows that *Dunaliella* ATPase activity required 100 $W \cdot m^{-2}$ for total saturation.

Although light-activated fructose-1,6-bisphosphatase activity was induced in *Dunaliella* with similar kinetics to those observed in leaves

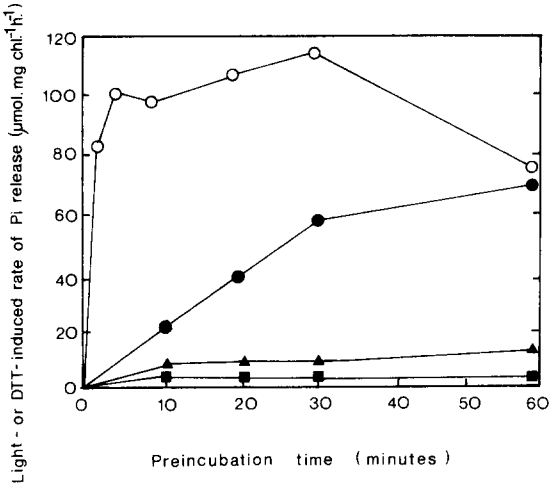

Fig. 7. Effect of incubation of intact algae with dithiothreitol (DTT) in the dark, compared with effect of illumination (90 $W \cdot m^{-2}$), on the rate of fructose 1,6-bisphosphate hydrolysis observed in subsequently lysed algae. ○, light-activated rates of fructose-1,6-bisphosphatase; ●, dark-adapted algae preincubated with 50 mM dithiothreitol; ▲, 20 mM dithiothreitol; ■, 10 mM dithiothreitol.

[24] and intact pea chloroplasts [17], a light intensity of 180 $W \cdot m^{-2}$ was required to fully induce steady-state fructose-1,6-bisphosphatase activity (Fig. 6). This contrasts with data obtained in intact chloroplasts and protoplasts (60 and 40 $W \cdot m^{-2}$, respectively [25]).

Experiments with methyl viologen (Fig. 3 for ATPase; data not shown for fructose-1,6-bisphosphatase) using the same rationale as previously used with intact chloroplasts [12] provide evidence that the light-induced enzyme activation is attributable to reduction by thioredoxin, thus indicating that the differences between reductant-controlled enzyme activation in *Dunaliella* and higher plants are quantitative rather than qualitative. The concentrations of methyl viologen required to inhibit enzyme activities were relatively high, but this might be expected in view of the barriers to its entry which exist between the external medium and stromal compartment of the intact algae.

Nevertheless, these results indicate a difference in the thiol-mediated regulation of CF_1-ATPase and fructose-1,6-bisphosphatase. Light-induction of fructose-1,6-bisphosphatase was totally prevented by addition of 10 mM methyl viologen to the preincubation medium prior to illumination (data not shown), whereas complete prevention of light-induced ATPase required 100 mM methyl viologen (Fig. 3). In addition, steady-state light-induced fructose-1,6-bisphosphatase was totally inhibited by addition of 10 mM methyl viologen in the light (data not shown). In contrast, addition of a 10-fold greater concentration to the preincubation medium in the light had no effect on steady-state light-activated ATPase (data not shown). These results can be explained in terms of a protective effect of the light induced $\Delta\tilde{\mu}_{H^+}$ upon the thiol-reduced ATPase, first proposed in [11]. Thus, light-activated CF_1 apparently remains thiol-reduced in the presence of a trans-thylakoid $\Delta\tilde{\mu}_{H^+}$, whereas fructose-1,6-bisphosphatase seems to be subject to a reversible thiol-regulation in the light [24], light-induced enzyme activity being dependent upon both reductant and oxidant concentration. This may also explain the requirement for higher light intensities and longer illumination times to fully induce enzyme activity in the case of fructose-1,6-bisphosphatase (cf. Figs. 4 and 6). Incubation of both intact chloroplasts [18] and protoplasts [1] with dithiothreitol in the dark has been used to mimic light-activation of fructose-1,6-bisphosphatase. Fig. 7 demonstrates that thiol-activation of fructose-1,6-bisphosphatase in the dark has been achieved in vivo in *Dunaliella*. In wheat protoplasts, this activation of stromal enzymes (as indicated by increase in fructose-1,6-bisphosphatase activity) led to a decrease of approx. 25% in the lag period of CO_2-dependent O_2 evolution upon subsequent measurement [1]. This compares with a 0–50% shortening observed in *Dunaliella*.

Attempts to assess the importance of enzyme activation by comparing absolute rates of catalysis, assayed under conditions purported to be physiological [13,17], to the rate of CO_2 fixation are difficult in the absence of any conclusive data regarding values of Mg^{2+} and substrate concentrations and pH which obtain in the illuminated stroma. Thus we have considered only the kinetics of thiol-mediated enzyme activation. The similarity of the kinetics of enzyme activation to those of CO_2-dependent O_2 evolution in *Dunaliella* (Figs. 4 and 6), allied with the observed dithiothreitol-induced decrease in the lag, support

the conclusion that enzyme activation limits the rate of CO_2 fixation under certain conditions during the induction period. Other studies of this nature have mostly led to the conclusion that enzyme activation proceeds too quickly to limit CO_2 fixation during the induction period [2,17,20,23]. With regard to this disparity it must be stressed that, while the pronounced similarity between the kinetics of enzyme activation and those of the onset of CO_2-dependent O_2 evolution (Figs. 4 and 6) observed in *Dunaliella* partly results from slower kinetics of enzyme activation in *Dunaliella* than in higher plants (cf. Fig. 4 with Fig. 2 of Ref. 23), it also results from the existence of shorter induction periods in *Dunaliella* than those generally seen in organelles and leaves from higher plants. Using chloroplasts and protoplasts from wheat displaying induction periods of 3–4 min, Leegood and Walker obtained evidence that it is the requirement for autocatalytic build-up of substrates rather than enzyme activation which is important during the lag period [1,2].

In conclusion, data presented here indicate that thiol-mediated enzyme activation contributes to the limitation of the rate of CO_2 fixation during the lag period of the green alga, *Dunaliella*. The role of enzyme activation during the steady state of CO_2 fixation is unclear. Although calculations of mass action ratios from measured leaf metabolite levels seemed to discount a limitation due to enzyme activation in the steady state [25,26], more recent evidence indicates a control over flux in the reductive pentose phosphate pathway by the extent of enzyme activation under conditions of light-limitation [27]. In support of this, Figs. 4 and 6 of this paper demonstrate that the rate of CO_2-dependent O_2 evolution observed after 5 min illumination increases in parallel with the steady-state extent of enzyme activation as the light intensity is increased from 0 to 105 and 0 to 180 $W \cdot m^{-2}$ (Figs. 4 and 6, respectively).

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