Biochimica et Biophysica Acta 894 (1987) 332–338
Elsevier

BBA 42650

Regulation of photosynthesis in isolated barley protoplasts: the contribution of cyclic photophosphorylation

Robert T. Furbank and Peter Horton

Research Institute for Photosynthesis, University of Sheffield, Sheffield (U.K.)

(Received 11 March 1987)

Key words: Photosynthesis; Photophosphorylation; Oxygen evolution; Chlorophyll fluorescence; (Barley protoplast)

The response of photosynthetic oxygen evolution and chlorophyll *a* fluorescence in isolated barley protoplasts to a change in light intensity (from approx. 1/10 saturating to saturating light levels) under saturating CO_2 concentrations was examined. Marked oscillations in the rate of O_2 evolution and chlorophyll fluorescence were observed, resembling those seen in leaves under similar conditions. The effect of antimycin A addition on O_2 evolution at various light intensities and at different times during photosynthetic induction was examined to determine the role of cyclic photophosphorylation during steady-state photosynthesis and during oscillatory behaviour. Antimycin (1 μM) addition reduced the photosynthetic rate by 50–75% but only at light intensities over 80–100 $\mu E \cdot m^{-2} \cdot s^{-1}$. Below this threshold light level, antimycin had no inhibitory effect. Thus, anitmycin had a progressively greater effect at higher light intensity. When antimycin was added at various points along the time course of photosynthetic induction, the rate of O_2 evolution was reduced to the same level regardless of the time of addition. Antimycin addition also had a marked effect on the oscillations in O_2 evolution observed after a transition in light intensity from 100 to 1000 $\mu E \cdot m^{-2} \cdot s^{-1}$, increasing the frequency and reducing the damping of the oscillations. Measurements of adenylate levels indicated that antimycin prevented a rapid rise in ATP/ADP ratio observed in the chloroplast compartment after the light transition. These results are discussed with reference to regulation of photosynthesis during a transition in light intensity and the limitation of photosynthesis by photophosphorylation under various environmental conditions.

Introduction

The existence of cyclic photophosphorylation in higher plants and its potential for ATP production have been well established [1,2]. The role of cyclic photophosphorylation in vivo appears to be in balancing the ATP requirements of photosynthesis relative to the requirements for reducing power [3,4]. The reductive pentose phosphate pathway requires 3 ATP and 2 NADPH per CO_2 fixed, while it appears from in vitro experiments that non-cyclic photophosphorylation alone (in the absence of a Q-cycle) could not provide this ATP/NADPH ratio (see Ref. 4). Furthermore, in non-steady-state conditions the ATP/NADPH demands of photosynthesis may vary between 1 and 1.7 [4]. Thus, flexibility in photophosphorylation may be vital to photosynthesis, particularly during times of high ATP demand, such as photosynthetic induction, when metabolite levels are autocatalytically increasing [1,4,5].

The operation of cyclic photophosphorylation during induction and during transitions in carbon flux through the reductive pentose phosphate

Abbreviation: Chl, chlorophyll.

Correspondence: R.T. Furbank, CSIRO, Division of Plant Industry, P.O. Box 1600, Canberra, ACT 2601, Australia.

pathway is far from clear. From P518 measurements and cytochrome turnover in isolated chloroplasts [1] it has been proposed that cyclic photophosphorylation is stimulated when the rate of electron transport is limited by NADP regeneration, particularly during induction. Thus, regulation of the proportion of ATP production occurring via this pathway may be regulated by the redox poise of a component of the electron-transport chain. There also appears to be regulation of the proportion of cyclic and noncyclic photophosphorylation via protein phosphorylation and consequently redox poising of plastoquinone [6,7], but the importance of this regulation to the flexibility of photosynthesis under different environmental conditions is not well established. Recently, regulation of photosynthesis has been examined by studying oscillations in the rate of photosynthesis (and in chlorophyll fluorescence) following transitions in light intensity and gas-phase composition [8–11]. Studies with leaves indicate that the relative rates of ATP consumption and production are important in delineating oscillatory behaviour [11,12], and it has been suggested that alterations in the rates of cyclic and linear electron flow may be of regulatory significance during oscillations [4]. Thus, it would be useful to study the role of cyclic photophosphorylation under such conditions where large-scale changes in the rate of phosphorylation must occur and in a homogeneous system where biochemical measurements can be more readily made. It has previously been shown that illumination of dark-adapted protoplasts results in an oscillatory transition in the rate of O_2 evolution, chlorophyll fluorescence and metabolite levels [13–15]. In this study, a transition in light intensity is used to generate multiple oscillations of the type seen in leaves. The contribution of cyclic photophosphorylation is assessed during photosynthetic induction, at various light intensities and during these oscillatory transients in photosynthesis. Antimycin A at low concentrations is used as an inhibitor of cyclic photophosphorylaton [16].

Materials and Methods

Barley (*Hordeum vulgar* L. var. Marko) was grown in a glass-house in trays of vermiculite moistened daily with Hoagland's solution. Illumination was provided by natural light supplemented by quartz-halide lamps. Growth temperature was 20–30 °C. The leaf tissue was harvested 10–14 days after germination and protoplasts isolated as described elsewhere [17], except that the digestion time was reduced to 2 h at 28 °C (this resulted in substantially higher photosynthetic rates with marginal loss in yield). Protoplasts were kept on ice in 0.5 M sorbitol, 1 mM $CaCl_2$ and 5 mM Mes at pH 6.0. Assays were carried out at 25 °C in 0.5 M sorbitol, 30 mM Tricine and 1 mM $CaCl_2$ at pH 7.6, at a chlorophyll concentration of 25 μg/ml in the presence of 5 mM $NaHCO_3$. Oxygen evolution was monitored using an O_2 electrode (Hansatech, King's Lynn, U.K.) coupled to an operational amplifier incorporating an electronic differentiator. This provided an immediate measure of the rate of O_2 evolution in real time.

Chlorophyll *a* fluorescence was measured using a system described by Horton [18] using a modulated measuring beam monitored by a photomultiplier tube coupled to a lock-in amplifier. This allowed the actinic light intensity to be altered without affecting fluorescence detection. Actinic light intensity was controlled by the use of neutral-density filters.

Separation of the chloroplast compartment from isolated protoplasts for denylate determinations was carried out using the silicone oil method [19] and routinely resulted in less than 10% contamination of chloroplast fractions by cytosolic metabolites (assessed by marker enzyme studies). The entire separation procedure was carried out under the same light intensity used for preincubation and the estimated quench time for this procedure was approx. 1 s. Adenylates were assayed using the luciferin/luciferase system [20]. All assays were performed at least 3 times using different protoplast preparations (unless otherwise indicated) and representative data are shown.

Results

The effect of low concentrations of antimycin A on CO_2-dependent O_2 evolution by isolated barley protoplasts has not previously been described. Thus it was necessary to determine the effects of this inhibitor during steady-state pho-

tosynthesis before applying it during oscillatory conditions. Although antimycin in nanomolar concentrations has been shown specifically to inhibit cyclic photophosphorylation in isolated chloroplasts, higher concentrations are known to uncouple photophosphorylation [16]. Fig. 1 shows that CO_2-dependent O_2 evolution in barley protoplasts was severely inhibited by antimycin at concentrations below 1 μM, the region where this inhibitor has been shown to act solely on cyclic photophosphorylation in isolated chloroplasts and not as a general uncoupler [16]. Also, the effective concentration of antimycin in the chloroplast compartment was probably far less than that in the medium due to non-specific binding of this inhibitor to cyotosolic proteins. The degree of antimycin inhibition of photosynthetic O_2 evolution was found to vary considerably with light intensity. Fig. 2 shows that 1 μM antimycin had little effect at light intensities below 100 $\mu E \cdot m^{-2} \cdot s^{-1}$ but had progressively greater effect at higher light intensities (75% inhibition at 500 $\mu E \cdot m^{-2} \cdot s^{-1}$). These data, along with those of figure 1, suggest that between 25 and 50% of maximum rates of photosynthesis can be supported at saturating light in the absence of cyclic photophosphorylation.

It has previously been proposed that cyclic photophosphorylation is of greater significance

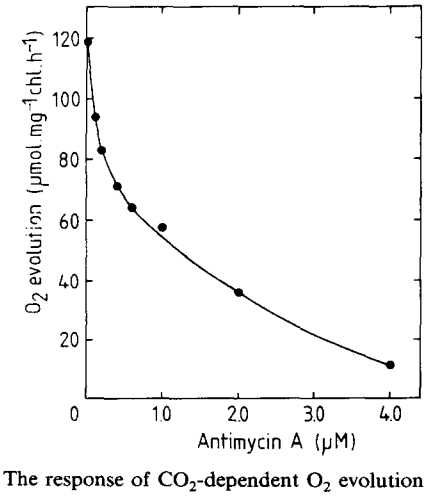

Fig. 1. The response of CO_2-dependent O_2 evolution in barley protoplasts to antimycin A concentration (added after a steady-state rate of photosynthesis was attained at a light intensity of 1800 $\mu E \cdot m^{-2} \cdot s^{-1}$).

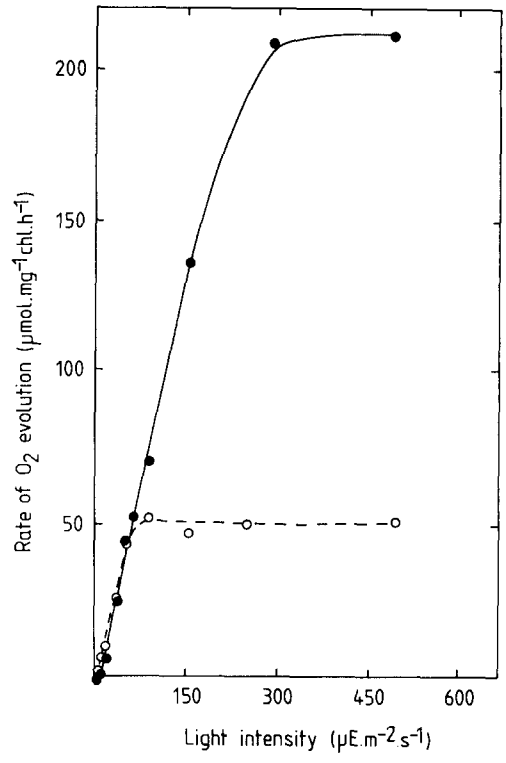

Fig. 2. Steady-state rates of CO_2-dependent O_2 evolution by barley protoplasts in the presence of 1 μM antimycin A (○) and in its absence (●), at a range of light intensities.

during inductive accumulation of chloroplast metabolites (see Refs. 1 and 4). To examine this hypothesis, antimycin (0.5 μM) was added to protoplasts in the presence of CO_2 after varying periods of illumination (at saturating light) (Fig. 3). During this induction period, intermediates of the reductive pentose phosphate pathway have been shown to increase in barley protoplasts and increasing levels of glycerate 3-phosphate correlating well with a transient in the rate of O_2 evolution [15]. Antimycin treatment during this period resulted in a reduction in the rate of O_2 evolution to the same level regardless of the amount of carbon fixed prior to antimycin addition. The concentration of antimycin used here was optimal for inhibition and further additions (up to 1 μM) had only a slight effect. It appears from these results that in a given protoplast preparation, only a low, fixed rate of photosynthesis is possible in the absence of cyclic photophosphorylation, irrespective of metabolite levels achieved at the time

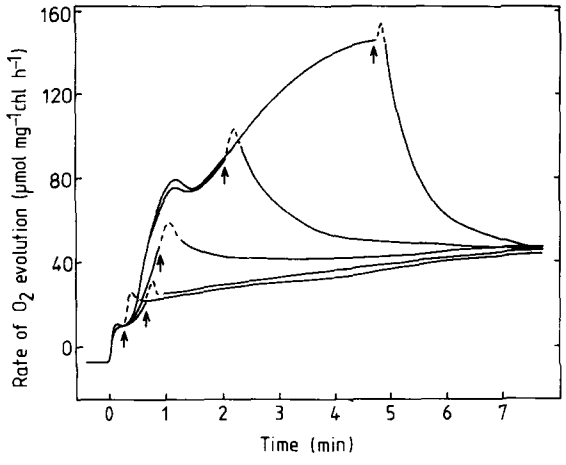

Fig. 3. The effect of antimycin A addition (0.5 μM) on CO_2-dependent O_2 evolution by barley protoplasts at various times during induction. Inhibitor addition is indicated by an arrow. The light (1000 $\mu E \cdot m^{-2} \cdot s^{-1}$) was turned on at time zero.

of addition. These data reinforce the conclusion that cyclic photophosphorylation is necessary in order to attain and sustain high rates of CO_2 fixation.

Oscillatory behaviour in the rate of O_2 evolution was observed to follow a pattern similar to that previously reported [13–15]. Fig. 4 shows, however, that this oscillation can take a more exaggerated form if a transition in light intensity is used rather than a dark-to-light switch. Isolated barley protoplasts were subjected to a transition in light intensity from sub-saturating to levels saturating for O_2 evolution (Fig. 4). The amplitude of the oscillation depended on the duration of preillumination in low light and the light intensity used. After 2 min preillumination at a light intensity of 100 $\mu E \cdot m^{-2} \cdot s^{-1}$ this effect appeared almost saturated. Transitions from light intensities above 200 $\mu E \cdot m^{-2} \cdot s^{-1}$ to saturating light (in excess of 600 $\mu E \cdot m^{-2} \cdot s^{-1}$ at 25 μg Chl per ml) resulted in progressively less oscillatory behaviour. As seen in leaves subjected to a dark-to-light transition [21,22], chlorophyll fluorescence oscillated in a manner broadly antiparallel to O_2 evolution, preceding it by 4–9 s.

The light transition shown in Fig. 4 was used to investigate the role of antimycin-sensitive photophosphorylation during a rapid change in flux through the reductive pentose-phosphate pathway (Fig. 5). Such an experiment is particularly interesting as the low light intensity found to be optimal for induction of oscillatory behaviour after a transition to high light corresponded to that required to saturate photosynthesis in the presence of antimycin (see Fig. 2). Barley protoplasts were illuminated at low light intensity (200 $\mu E \cdot m^{-2} \cdot s^{-1}$) until steady state photosynthesis was reached (as in Fig. 4), antimycin was added and the light intensity increased without 30 s (to over 1000 $\mu E \cdot m^{-2} \cdot s^{-1}$ in these experiments). The rates of O_2 evolution obtained were compared with protoplasts from the same preparation treated identically in the absence of antimycin. The presence of antimycin, despite having little effect on the rate of photosynthesis at low light, caused multiple, rapid oscillations in the rate of O_2 evolution after the transition to high light (Fig. 5). In the absence of antimycin, the period of oscillation was approx. 35 s, while in the presence of antimycin, this period reduced to about 12 s and three damped oscillations were observed rather than two. How-

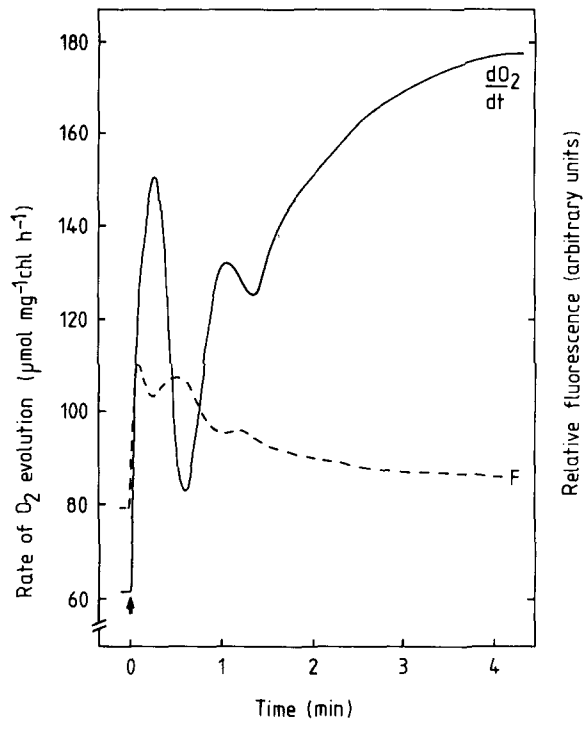

Fig. 4. The effect of a transition in light intensity from 100 $\mu E \cdot m^{-2} \cdot s^{-1}$ to saturating light on the rate of CO_2-dependent O_2 evolution by barley protoplasts. Protoplasts were incubated for 2 min at low light. Chlorophyll *a* fluorescence (broken line) was monitored simultaneously with O_2 evolution (solid line).

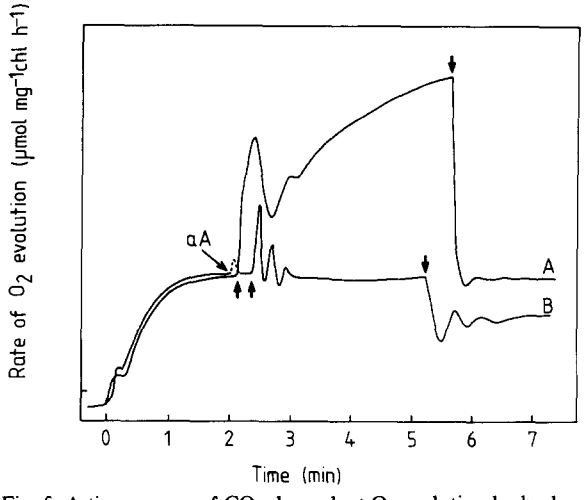

Fig. 5. A time-course of CO_2-dependent O_2 evolution by barley protoplasts during a transition from 200 to 1200 $\mu E \cdot m^{-2} \cdot s^{-1}$. In trace A no inhibitor was added, while in trace B, 1 μM antimycin was added as indicated. An increase in light intensity is indicated by ↑ and a decrease by ↓.

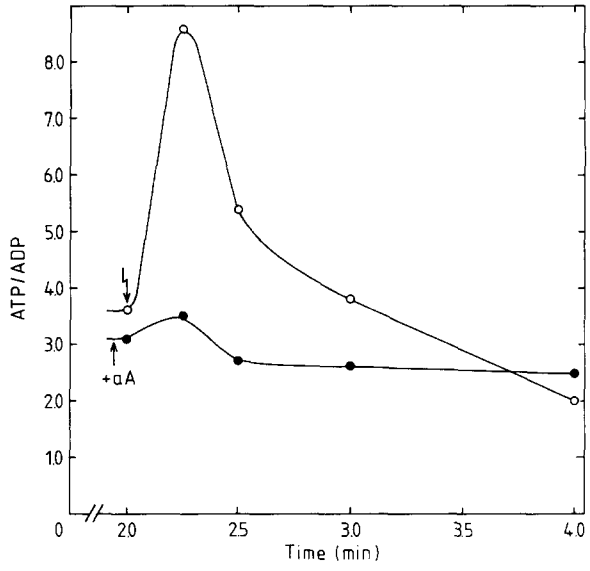

Fig. 6. Changes in the ATP/ADP ratio of the chloroplast compartment of barley protoplasts following a transition in light intensity from 200 to 1800 $\mu E \cdot m^{-2} \cdot s^{-1}$ in the presence of 1 μM antimycin (●) and with no inhibitor present (○). Protoplasts were preincubated at low light for 2 min prior to the increase in light. Total adenylates remained constant (100–115 $nmol \cdot mg^{-1} \cdot Chl^{-1}$) throughout the time-course.

ever, the final rate of photosynthesis in high light did not exceed that seen at the lower light intensity. An oscillation was also induced by the transition back to low light with antimycin present. The new rate of O_2 evolution attained at low light had decreased after illumination in high light with antimycin, presumably due to photoinhibitory damage.

The action of antimycin in Fig. 5 was presumably due to impairment of photophosphorylation, causing not only restriction of photosynthesis in high light but a severe metabolic imbalance. This hypothesis was investigated by mesuring levels of ATP and ADP in the chloroplast compartment of the protoplasts at various points following the transition in light intensity (Fig. 6). Absolute adenylate levels varied considerably between preparations, as did ATP/ADP ratios (the former between 50 and 150 nmol per mg Chl, the latter between 1.8 and 4, determined in six preparations at low light). However, within a single protoplast preparation, the means of four separate determinations varied less than 12%. Thus, the data shown are the averages of four determinations on a single protoplast preparation. In the absence of antimycin, the ATP/ADP ratio rose from 3.5 to 8.5 within 15 s after the transition from 200 $\mu E \cdot m^{-2} \cdot s^{-1}$ to 1800 $\mu E \cdot m^{-2} \cdot s^{-1}$. However, within 2 min this ratio had fallen to a value below that measured in low light. In the presence of 1 μM antimycin, this transition in light intensity produced only a 10% increase in the ATP/ADP ratio.

Discussion

The effect of antimycin on the rate of O_2 evolution during induction and at various light intensities suggests that cyclic photophosphorylation is necessary to support maximum rates of steady state photosynthesis at high light. This has previously been suggested from work with isolated chloroplast systems [1,23,24], but not convincingly in vivo. It is difficult to extrapolate data obtained with isolated chloroplasts to the in vivo case, as the effect of antimycin on CO_2-dependent O_2 evolution vary enormously with assay conditions [5]. This problem is partially overcome in the protoplast system where the chloroplasts are photosynthesising in situ without added P_i or metabolites. The importance of cyclic photophosphorylation during photosynthetic induction cannot be refuted or supported by the results shown here but it is evident that the provision of ATP during the

induction period is not the only role for this process in vivo. If this simple situation were the case, the addition of antimycin at various points along the time-course of induction would halt the increase in rate but not cause inhibition of the rate at that point in time.

A requirement for cyclic photophosphorylation during steady-state photosynthesis is supported by the progressively greater inhibition of photosynthesis by antimycin at higher light intensities. At low light intensity there is no inhibition of photosynthesis, possibly due to the capacity of pseudocyclic photophosphorylation to provide flexibility in the production of ATP. Above a threshold light intensity (or carbon flux rate during induction) there may be insufficient capacity for alternative ATP-producing mechanisms to substitute for cyclic photophosphorylation. This implies that, although non-cyclic electron flow can theoretically satisfy a large proportion of the demand, increasing the quantum flux in the absence of cyclic electron flow is ineffective in stimulating photosynthesis. Also, at high light intensity, the electron-transport chain is more reduced (Q_A is almost totally reduced at the light intensity used here; data not shown), a situation which would promote cyclic electron transport. It must be remembered, however, that although antimycin A has been shown to selectively inhibit cyclic photophosphorylation in chloroplasts at the concentrations used here, this inhibitor also blocks mitochondrial electron transport. There is a great deal of controversy over the role of mitochondrial respiration in the light, but it seems unlikely that inhibition of respiration in the presence of saturating CO_2 and high light could produce the effects observed here.

Examination of chloroplast adenylate levels during this transition in light intensity indicated that the major effect of antimycin was a suppression of the rise in ATP/ADP ratio seen immediately after the change to high light. The ATP/ADP ratios in both treatments were, however, similar in the steady state. This suggests that cyclic photophosphorylation was largely responsible for this ATP 'burst'. In the antimycin-treated preparation, the rate of O_2 evolution showed a series of sustained, rapid oscillations about the rate seen at the lower light intensity. When illumination was reduced to the previous level, the antimycin-treated sample showed marked oscillatory behaviour while the control did not. Such an exacerbation of the oscillations by antimycin and the increase in the frequency of oscillations suggest that cyclic photophosphorylation exerts a damping influence during transitions in light intensity, presumably by providing flexibility in ATP production relative to NADP reduction. This is particularly interesting; in view of observations intact leaves also show a transient overshoot in the rate of photosynthesis, often above the steady-state rate, on illumination in high CO_2 and light [22] or after a transition from low to high light [10]. In the latter case, a concomitant increase in ATP levels and a decrease in the ratio of glycerate-3-phosphate to triose phosphate is seen, attributed to 'excess' electron-transport capacity, unused at steady state [10]. This may, however, be indicative of a stimulation of cyclic photophosphorylation by the sudden reduction of the electron-transport chain at high light.

It is interesting to note that the oscillations in O_2 evolution observed here could be manipulated solely by changing the capacity of the thylakoid for photophosphorylation. An explanation for oscillatory phenomenona of this type has previously been proposed (see Refs. 4, 14 and 22). When a change in photosynthetic carbon flux occurs in response to a transition in gas phase or light intensity, pools of the reductive pentose-phosphate pathway are adjusted concurrent with an adjustment in the rate of ATP consumption and production [4,22,12]. Competition between the two kinase reactions of the Calvin cycle for ATP after a pulse of carbon through the cycle causes a temporatory 'bottle-neck' at the glycerate-3-phosphate kinase reaction. This theory agrees well with measurements of metabolites in leaves [12] and protoplasts [15]. The origin of the perturbation inducing the oscillation (i.e., gas phase or light intensity) obviously affects the order of metabolic events and it has been proposed that cytosolic processes might contribute to photosynthetic oscillations [9,11,25] by limiting P_i supply to the chloroplast. After a transition from low to high light for example, there may be a delay before the rate of sucrose synthesis can increase to provide an adequate P_i supply to the chloroplast.

This would contribute to the restriction of ATP supply and exacerbate the oscillation. With regard to this, the observation that the effect of antimycin on intact chloroplasts is very P_i dependent [5] may be relevant. It has been demonstrated that antimycin inhibition is most severe with low P_i levels in the reaction medium, while at superoptimal P_i, antimycin may even reverse P_i inhibition [5]. It is possible that the progressively greater inhibition of O_2 evolution at higher light intensities observed here is analogous to the isolated chloroplast situation, i.e., chloroplast P_i in vivo may be lower at high light. Also, the rapid decline in chloroplast ATP seen in protoplasts after 1 min illumination in high light (Fig. 6) could be due to exhaustion of stromal P_i.

It has been shown using a mathematical model of chloroplast carbon metabolism, that restriction of the supply of P_i to the chloroplast stroma could result in an oscillation in photosynthesis of the type seen in leaves [25]. This supported by the observation that mannose feeding of leaves (which sequesters cytosolic P_i) increases oscillatory behaviour [26]. Previous attempts to model such oscillations using solely chloroplast ATP supply and demand as variables have resulted in oscillations of higher frequency than tose seen with leaves [27]. These oscillations are more similar to those seen in protoplasts in the presence of antimycin. In a recent computer simulation of photophosphorylation [28], inhibition of cyclic photophosphorylation was seen to produce rapid oscillations in photosynthesis identical to those seen here (Fig. 5). These simulated oscillations did not, unlike the slower ones seen under control conditions, involve a regulatory step in sucrose synthesis. Also, the oscillations in the model disappear if the ATP/NADPH stoichiometry for linear electron flow is raised to 1.5, confirming that stability of the photosynthetic system requires a close regulatory control between ATP and NADPH supply and demand. The results shown here support the hypothesis that sustained photosynthetic oscillations can result entirely from chloroplast reactions.

Acknowledgements

We wish to thank Prof. D.A. Walker for constructive criticism of this work.

References

1 Slovacek, R.E., Crowther, D. and Hind, G. (1980) Biochim. Biophys. Acta 592, 495–505
2 Woo, K.C., Gerbaud, A. and Furbank, R.T. (1983) Plant Physiol. 72, 321–325
3 Heber, U. (1973) Biochim. Biophys. Acta 304, 140–152
4 Horton, P. (1985) in Photosynthetic Mechanisms and the Environment (Baker, N.R. and Barber, J., eds.), pp. 135–187, Elsevier, Amsterdam
5 Walker, D.A. (1976) in The Intact Chloroplast (Barber, J., ed.), pp. 235–278, Elsevier, Amsterdam
6 Horton, P. (1983) FEBS Lett. 152, 47–52
7 Allen, J.F. (1983) Trends Biochem. Sci. 8, 369–373
8 Sivak, M.N., Prinsley, R.T. and Walker, D.A. (1983) Proc. R. Soc. Lond. B217, 393–404
9 Leegood, R.C. and Furbank, R.T. (1986) Planta 168, 84–93
10 Stitt, M. (1986) Plant Physiol. 81, 1115–1122
11 Sivak, M.N. and Walker, D.A. (1986) in Biological Control of Photosynthesis (Marcelle, R., Clijsters, H. and Van Pouche, M., eds.), pp. 1–31, Martinus Nijhoff, Dordrecht
12 Furbank, R.T. and Foyer, C.H. (1986) Arch. Biochem. Biophys. 246, 240–244
13 Quick, W.P. and Horton, P. (1984) Proc. R. Soc. Lond. B220, 361–370
14 Quick, W.P. and Horton, P. (1984) Proc. R. Soc. Lond. B220, 371–382
15 Quick, W.P. and Horton, P. (1986) Biochim. Biophys. Acta 849, 1–6
16 Moss, D.A. and Bendall, D.S. (1984) Biochim. Biophys. Acta 767, 389–395
17 Edwards, G.E., Robinson, S.P., Tyler, N.J.C. and Walker, D.A. (1978) Plant Physiol. 62, 313–319
18 Horton, P. (1983) Proc. R. Soc. Lond. B217, 405–416
19 Robinson, S.P. and Walker, D.A. (1979) Arch. Biochem. Biophys. 196, 319–323
20 Carver, C., Hope, A. and Walker, D.A. (1982) Biochim. Biophys. Acta 210, 273–276
21 Walker, D.A., Horton, P., Sivak, M.N. and Quick, W.P. (1983) Photobiochem. Photobiophys. 5, 35–39
22 Walker, D.A., Sivak, M.N., Prinsley, R.T. and Cheesbrough, J.K. (1983) Plant Physiol. 73, 542–549
23 Fejzo, J., Plesnicar, M. and Cerovic, Z.G. (1986) Proc. R. Soc. Lond. B. 228, 471–482
24 Furbank, R.T. (1982) Ph.D. Thesis, Australian National University, Canberra
25 Laisk, A. and Walker, D.A. (1986) Proc. R. Soc. Lond. B227, 281–302
26 Harris, G.C., Cheesbrough, J.K. and Walker, D.A. (1983) Plant Physiol. 71, 108–111
27 Giersch, C. (1986) Arch. Biochem. Biophys. 245, 263–270
28 Horton, P. and Nicholson, H. (1987) Photosyn. Res. 12, 129–143