Biochimica et Biophysica Acta 894 (1987) 552–561
Elsevier

BBA 42685

Regulation of photosynthesis in isolated spinach chloroplasts during orthophosphate limitation

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(Received 22 June 1987)

Key words: Photophosphorylation; Phosphate limitation; Calvin cycle; Thylakoid ΔpH; Photosynthesis regulation; (Spinach chloroplast)

The stromal concentration of orthophosphate in intact spinach chloroplasts (prepared in the absence of orthophosphate or pyrophosphate but supplied with both in the reaction medium) fell from a value of approx. 20 mM in the dark to a steady-state concentration of approx. 8 mM in the light. Chloroplasts illuminated in the absence of orthophosphate or pyrophosphate showed a similar trend. However, in this situation the stromal inorganic phosphate (P_i) concentration rapidly decreased from approx. 10 mM in the dark to a constant steady-state concentration of between 1.5 and 2.5 mM in the light. This P_i concentration was not further diminished (even though CO_2-dependent O_2 evolution had ceased) and was therefore considered to be stromal orthophosphate not freely available to metabolism. In the P_i-deficient chloroplasts the rate of photosynthesis declined rapidly after 1–2 min in the light such that CO_2-dependent O_2 evolution ceased with 5 min of the onset of illumination. The decline in O_2 evolution was accompanied by an increase in the transthylakoid ΔpH (as measured by 9-aminoacridine fluorescence quenching) and in the high-energy state, non-photochemical component of chlorophyll fluorescence quenching (q_E). Measurements of stromal metabolite concentrations showed that the ATP/ADP ratio was decreased in the P_i-deficient chloroplasts relative to chloroplasts illuminated in the presence of P_i. The stromal concentration of glycerate 3-phosphate was comparable in the P_i-deficient chloroplasts and those to which P_i had been supplied. Chloroplasts which were illuminated in P_i-free media showed a large accumulation of ribulose-1,5-bisphosphate relative to those supplied with P_i, suggesting inhibition of ribulose-1,5-bisphosphate carboxylase under these conditions. When P_i was added to chloroplasts illuminated in the absence of P_i, both non-photochemical quenching (q_E), photochemical quenching (q_Q) and ΔpH increased. This suggests that electron transport was not limited by inability to discharge transthylakoid ΔpH. These observation are consistent with the hypothesis that P_i limitation results in decreased ATP production by the thylakoid ATP synthase. The data presented here show that there are multiple sites of flux control exerted by low stromal P_i in the chloroplast. At least three factors contribute to the inhibition of photosynthesis under phosphate limitation: (1) there appears to be a direct effect of P_i on the energy-transducing system; (2) there is direct inhibition of the Calvin cycle decreasing the ability of the pathway to act as a sink for ATP and NADPH; and (3) feedback inhibition of primary processes occurs either via ΔpH or the redox state of electron carriers. However, ΔpH does not

Abbreviations: P_i, inorganic phosphate; Mes, 4-morpholineethanesulphonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; PP_i, inorganic pyrophosphate; PS II, Photosystem II; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Chl, chlorophyll.

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appear to be a limiting factor, but rather an inability to regenerate NADP as electron acceptor is suggested. The addition of DCMU to chloroplasts during illumination in the absence of P_i for periods of up to 10 min showed that there was very little loss of variable fluorescence despite a 60% reduction in the capacity for O_2 evolution. This would suggest that photoinhibitory damage to Photosystem II was not the major cause of the inhibition of photosynthesis observed with low P_i.

Introduction

In the 1960s it was shown that isolated chloroplasts required a supply of orthophosphate in order to evolve O_2 with CO_2 as a hydrogen acceptor [1]. Subsequently, higher concentrations of P_i were found to be inhibitory [2]. Moreover, this inhibition could be reversed by certain Calvin-cycle intermediates [2]. These and other results led to the concept of obligatory exchange between internal sugar phosphates and external P_i [3] and to experiments which led to the characterisation of the P_i-translocator [4]. It was also supposed that what occurred in vitro might reflect an in vivo situation in which the P_i requirement of the photosynthesising chloroplasts might be met by P_i released during the utilisation of triose phosphate, exported from the chloroplasts, in cytosolic sucrose synthesis [5,6]. In accord with this theory, cytosolic orthophosphate concentration has been shown not only to affect the rate of photosynthesis in vivo [7], but also to influence the distribution of metabolites between starch and sucrose [8,9]. A major uncertainty has, however, persisted for many years [5–7,10] and this may be most simply addressed by asking how the chloroplast perceives that it is becoming P_i limited. It is obvious, of course, that the stromal P_i concentration might fall low enough to limit ATP synthesis and consequently inhibit CO_2 fixation. Limitation of the ATPsynthase by low P_i in vivo is supported by observations of increased thylakoid energisation, as evidenced by high non-photochemical chlorophyll fluorescence quenching, in the presence of P_i sequestering agents [11] and after a transition to 2% O_2 [12]. One possible weakness of this hypothesis is that the K_m P_i of the ATPase determined in vitro is at maximum approx. 0.6 mM [13], whereas the measured chloroplast P_i concentration in the light is much higher (between 8 and 35 mM). However, it is probable that a proportion of chloroplast P_i is unavailable to the ATP-synthase because of binding by ribulose 1,5-bisphosphate carboxylase [14–16] or shielding of the ATP-synthase by stromal protein [15,16]. Robinson and Giersch [17] have reported that approx. 1 mM of the P_i in the stroma is associated with thylakoid membranes as a non-metabolic pool. In addition to effects on photophosphorylation low [P_i] may have a direct effect on carbon metabolism, for example on the activity of ribulose-1,5-bisphosphate carboxylase activity [18,19].

In this study simultaneous measurements of chlorophyll *a* fluorescence, 9-aminoacridine fluorescence and O_2 evolution, coupled to metabolite measurements, have been used to reappraise the basis of inhibition and regulation of photosynthesis at low and high P_i concentrations in isolated chloroplasts.

Methods

Isolated chloroplasts were prepared from glass-house grown *Spinacia oleracea* L. cv virtuosa [20]. Sorbitol (0.33 M) in 10 mM Mes/KOH buffer (pH 6.5) was used as a grinding medium to avoid contamination of the preparation with pyrophosphate and P_i.

CO_2-dependent O_2 evolution, chlorophyll *a* fluorescence and 9-aminoacridine fluorescence were measured [21,22] in a medium containing intact chloroplasts (50 μg per ml chlorophyll), 0.33 M sorbitol, 2 mM $MgCl_2$, 2 mM EDTA, 50 mM Hepes/KOH (pH 7.6), 10 mM $NaHCO_3$, 3 μM 9-aminoacridine and 2000 U of catalase in the absence or presence of added P_i alone or 5 mM PP_i and 0.5 mM P_i. Methyl viologen and nigericin were added as indicated. The components of chlorophyll *a* fluorescence quenching were elucidated using the light-doubling technique [20,21]. Actinic illumination (700 μmol quanta $\cdot m^{-2} \cdot s^{-1}$ red light, defined by a Balzers RG 610 and an Ealing 660 short-pass filter) was provided by a quartz projector lamp. A saturating pulse of light (2000

μmol quanta $\cdot m^{-2} \cdot s^{-1}$ defined by the same filters as the actinic light) was flashed for a duration of 1.5 s every 7 s during illumination to reduce Q_A. This flash frequency and duration saturated Q_A but did not significantly affect the steady-state variable fluorescence yield or the rate of O_2 evolution. Chlorophyll *a* fluorescence was monitored using a Hansatech modulated fluorescence detection system (Hansatech, Kings Lynn, U.K.) with the detection filters replaced by a Balzers RG 715. Fluorescence from 9 aminoacridine was monitored using a modulated system [22]. Values for q_Q and q_E were calculated from the fluorescence yield after a saturating flash of light according to Schreiber et al. [23]. Oxygen was measured with a Clark-type electrode (Hansatech, Kings Lynn, U.K.). All instruments were interfaced to a BBC Master Series microcomputer (Acorn Computers, Cambridge, U.K.).

Measurements on thylakoids were performed in a reconstituted chloroplast system in which intact chloroplasts were osmotically shocked by addition to 0.5 ml of water containing 2 mM $MgCl_2$. The components of the assay medium were 0.33 M sorbitol, 5 mM dithiothreitol, 1 mM EDTA, 10 mM KCl, 11 mM $MgCl_2$, 4 mM ascorbate and 50 mM Hepes/KOH buffer (pH 7.9).

Metabolite measurements were made with intact chloroplasts illuminated in the reaction medium (above) except that the reaction volume was 2.5 ml. Samples (200 μl) removed at 1 min intervals and immediately centrifuged through 100 μl silicone oil [24] into 10% $HClO_4$ to separate chloroplasts from the medium. For the samples taken in the light the entire extraction process was carried out in the light (700 μmol quanta $\cdot m^{-2} \cdot s^{-1}$). 20 replicate samples were taken for each time point and pooled (to obtain the equivalent of 200 μg chlorophyll). Glycerate 3-phosphate, triose phosphate and fructose 1,6-bisphosphate were measured enzymically, adenylates were determined by the luciferase technique, ribulose 1,5-bisphosphate was determined radiometrically [25] and P_i was determined colorimetrically [26].

To measure the activity of ribulose 1,5-bisphosphate carboxylase samples (50 μl) were removed from the cuvette and immediately assayed in a 0.5 ml volume of 100 mM tricine pH 8.1, 20 mM $MgCl_2$, 4 mM ribulose 1,5-bisphosphate and 10 mM $NaH^{14}CO_3$ (37 Gbq $\cdot$ mol^{-1}). Aliquots were removed after 15 and 30 s, added to ion formic acid, evaporated to dryness and radioactively assayed in a liquid scintillation counter.

Results

The effect of P_i on induction

The effects of $[P_i]$ on CO_2-dependent O_2 evolution, chlorophyll *a* fluorescence quenching and 9-aminoacridine fluorescence quenching in isolated spinach chloroplasts are shown in Fig. 1. The duration of the induction phase of photosynthesis increased as the P_i concentration was increased. The P_i optimum for O_2 evolution was

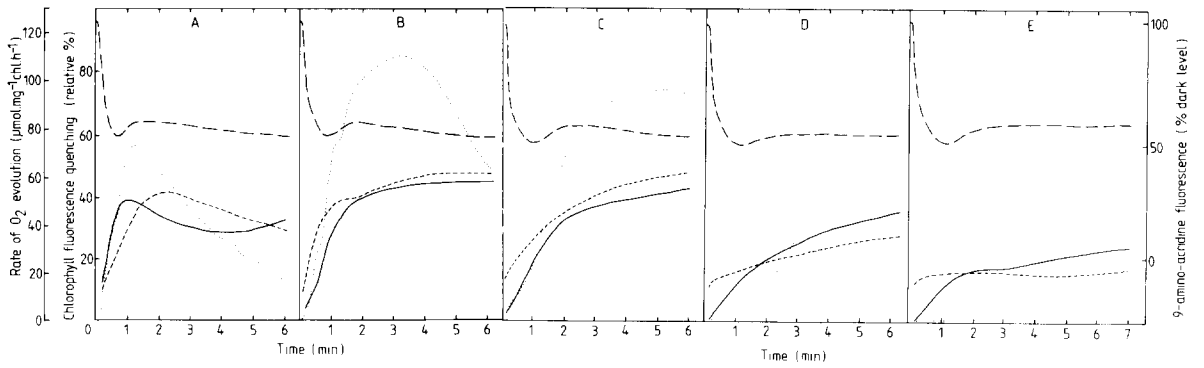

Fig. 1. Time-courses of CO_2-dependent O_2 evolution (·····), q_Q (- - - - -), q_E (———) and 9-aminoacridine fluorescence quenching (— — —) in isolated chloroplasts illuminated in the presence of various P_i concentrations. P_i was added prior to illumination at the following concentrations: 0 (1A), 0.1 mM (1B), 0.3 mM (1C), 1.0 mM (1D) and 2.0 mM (E). A decrease in 9-aminoacridine fluorescence is indicative of an increase in ΔpH.

approx. 0.2 mM P_i. Almost total inhibition of O_2 evolution occurred at 2 mM P_i.

The effect of P_i availability on chlorophyll *a* fluorescence quenching was monitored during the induction period of photosynthesis. The components of chlorophyll *a* fluorescence analysed were q_Q (photochemical quenching associated with the oxidation of Q_A, the primary electron acceptor of PS II) and q_E (non-photochemical quenching of chlorophyll *a* fluorescence). These components were resolved by applying flashes of light in order to fully reduce Q_A (which quenches fluorescence only in its oxidised state). The fluorescence quenching, remaining after Q_A is fully reduced, is defined as non-photochemical quenching (the majority of which appears to be due to the high-energy state of the thylakoid membrane and consequently is related to the magnitude of the ΔpH (see Refs. 20 and 23). Photochemical quenching is defined by the increase in chlorophyll fluorescence occurring during application of the saturating flash. The kinetics of chlorophyll fluorescence quenching were most complex at low [P_i] when a series of complimentary transients were seen (Fig. 1). Similar transients were also observed in 9-aminoacridine fluorescence (Fig. 1). At low [P_i], a large proportion of q_E formed within the first minute of illumination. This subsequently declined to a minimum and rose again as P_i became exhausted. Photochemical quenching also developed rapidly following the onset of illumination at low P_i but decreased as P_i became exhausted.

The maximum q_E value was increased by over 60% when the concentration of added P_i was increased from 0 to 0.2 mM and then declined above this concentration. However, 9-aminoacridine fluorescence quenching remained constant over the this P_i range, suggesting that the thylakoid ΔpH did not change in response to [P_i]. There is, therefore, discrepancy between q_E and 9-aminoacridine fluorescence.

The changes in q_Q paralleled the changes in O_2 evolution, increasing between 0 and 0.2 mM P_i and declining at higher [P_i]. During the induction period, however, the relationship between the rate of O_2 evolution and q_Q was not linear.

The changes in the stromal concentrations of key photosynthetic metabolites during the induction period through to steady-state photosynthesis were measured in intact chloroplasts in the presence of 5 mM PP_i and 0.5 mM P_i and in the absence of these compounds (Fig. 2). In the P_i-deficient chloroplasts, the P_i content of the stroma fell from a dark value of 10 mM (assuming a stromal volume of 25 μl per mg chlorophyll) to 2

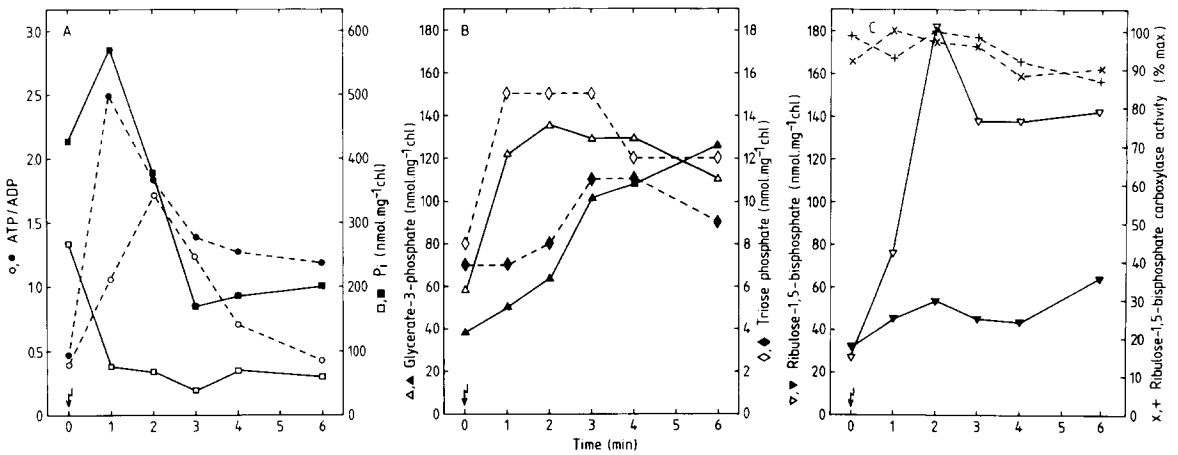

Fig. 2. Measurements of photosynthetic metabolites (A, B, C) and ribulose 1,5-bisphosphate carboxylase activity in spinach chloroplasts illuminated with 10 mM $NaHCO_3$ in the absence of P_i (open symbols) and in the presence of 5 mM PP_i and 0.5 mM P_i (closed symbols). ATP/ADP ratios (●, ○), P_i (■, □), triose phosphate (◆, ◇), glycerate 3-phosphate (▲, △) and ribulose 1,5-bisphosphate (▼, ▽) levels are shown. Ribulose 1,5-bisphosphate carboxylase activity in P_i limited (×) and P_i supplied (+) chloroplasts was assayed immediately in samples removed from the O_2 electrode cuvette.

mM within 1–2 min of illumination. During this period, the ATP/ADP ratio reached a maximum and then declined to dark values around 0.5 (Fig. 2). The rates of O_2 evolution, q_Q and q_E all reached maxima within 2 min illumination but q_E gradually increased after 3 min in the light (Fig. 3A and B). In chloroplasts supplied with P_i, the ATP/ADP ratio increased to a maximum of 3 during the induction period and subsequently decreased to a constant value of 1.25 while the stromal P_i fell from a dark concentration of 20 mM to a steady-state concentration of approx. 8 mM. In these chloroplasts, the rate of O_2 evolution, q_Q and q_E all rose gradually over a 3–4 min period (Fig. 3C and D). In the P_i-deficient chloroplasts, triose phosphates and glycerate 3-phosphate increased during the first minute of illumination to higher values that those in chloroplasts supplied with P_i. Ribulose 1,5-bisphosphate concentration in low P_i chloroplasts became 6-fold higher than in the control chloroplasts within 2 min of illumination suggesting a limitation of carbon metabolism at this step. No changes in the measured activity of ribulose 1,5-bisphosphate carboxylase were observed in these chloroplasts during the transition from darkness to light or during illumination. The ribulose-1,5-bisphosphate carboxylase activity present in the chloroplasts illuminated in the absence of P_i was comparable to that in chloroplasts to which P_i was supplied (Fig. 3C). In some experiments stromal fructose 1,6-bisphosphate concentration also increased significantly during the induction period when P_i was omitted from the reaction mixture (data not shown).

Effects of P_i supply on coupled electron flow

The electron acceptor, methyl viologen and the uncoupler, nigericin were used in further examination of the effects of P_i limitation on photophosphorylation and electron transport in intact chloroplasts. Fig. 4A shows the effect of a low concentration of the uncoupler nigericin (1 nM) added to intact chloroplasts (illuminated in the absence of added P_i) when photosynthetic O_2 evolution had ceased. The uncoupler decreased 9-

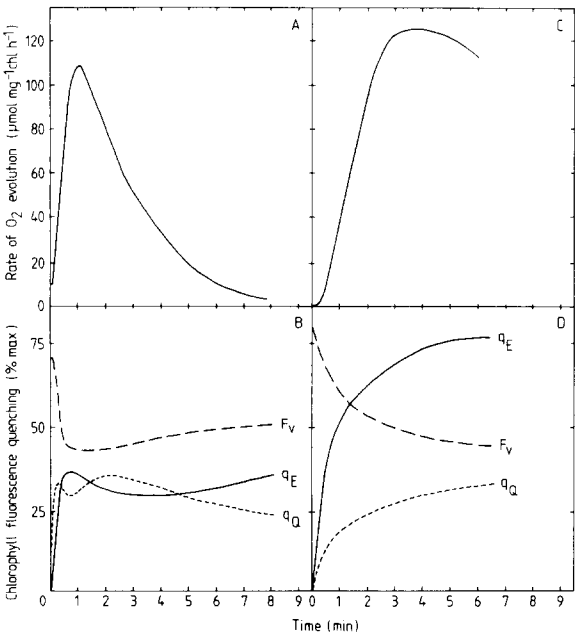

Fig. 3. (A and C) The rate of O_2 evolution (———). (B and D) q_Q (- - - - -) q_E (———) and variable fluorescence, F_v, — — — —) measured with the same chloroplast preparation as that in Fig. 2. Chloroplasts were illuminated in the absence of P_i (A and B) and in the presence of 5 mM PP_i and 0.5 mM P_i (C and D).

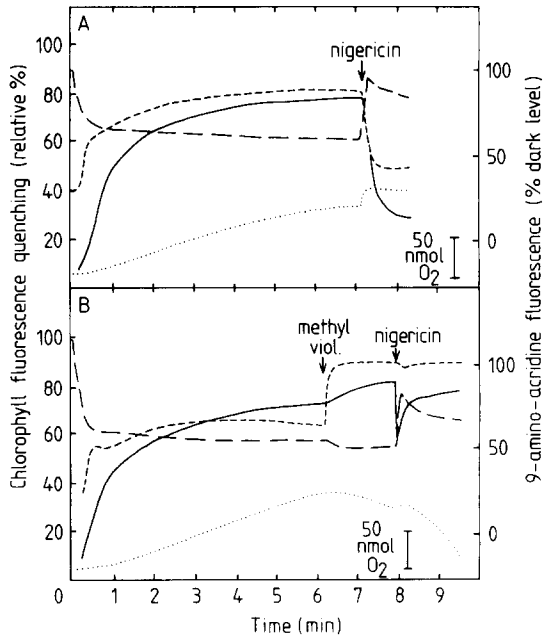

Fig. 4. The effect of nigericin addition on O_2 evolution (· · · · · ·), q_Q (— — —), q_E (———) and 9-aminoacridine fluorescence (— — —) in intact chloroplasts illuminated in the absence of added P_i (A). Also shown (B) is the effect of methyl viologen addition (0.1 mM) and subsequent nigericin addition under the same conditions.

aminoacridine fluorescence (and therefore relaxed the thylakoid ΔpH) and q_E also decreased. The addition of nigericin elicited a transient burst of O_2 evolution (Fig. 4A).

When the electron acceptor methyl viologen was added to intact chloroplasts which had ceased to evolve O_2 following illumination in the absence of P_i, there was an immediate increase in q_Q, q_E and ΔpH and a low rate of O_2 uptake was initiated. The subsequent addition of nigericin caused a rapid collapse in both ΔpH and q_E and the rate of O_2 evolution was enhanced (Fig. 4B). Although it is presumptuous to assume direct cause and effect relationships in complex systems, these results show that under P_i limitation, the addition of methyl viologen can yield even larger ΔpH values than those supported by endogenous NADP pools. This suggests that electron flow is not severely limited by ΔpH in these circumstances. To examine this phenomenon further, P_i was added to P_i-depleted chloroplasts during illumination. Fig. 5 shows the effect of the addition of 0.1 mM P_i on O_2 evolution, chlorophyll *a* fluorescence quenching and 9-aminoacridine fluorescence quenching in spinach chloroplasts illuminated for 10 min in the absence of added P_i. Following illumination the initial fluorescence kinetics were similar to those shown in Fig. 1. After 4 min illumination, both q_E and 9-aminoacridine fluorescence quenching increased substantially. After 10 min illumination, P_i was added and q_E, q_Q and 9-aminoacridine fluorescence quenching increased and O_2 evolution was initiated. The rate of O_2 evolution attained after the addition of P_i was lower than that produced initially by the onset of illumination or with 0.1 mM P_i present in the reaction medium.

The loss of photosynthetic capacity caused by illumination in the absence of P_i was studied by measuring the O_2 evolution rate obtained when 0.1 mM P_i was added to chloroplasts following illumination in P_i-free media for varying periods of time (Table I). Maximal rates were obtained when P_i was added after 2 min illumination. Periods of illumination greater than 2 min in the absence of P_i progressively decreased photosynthetic capacity. For example, a 30% decrease in maximum rate was observed after 5 min and when P_i was added after 10 min illumination, subsequent CO_2-dependent O_2 evolution declined by approx. 60%. Loss of variable chlorophyll *a* fluorescence has previously been correlated with photoinhibitory damage to the photosynthetic apparatus [27], a process which could contribute to the decline in photosynthesis occurring during illumination in the absence of P_i. To measure the degree of photoinhibition occurring in the chloroplasts illuminated in the absence of P_i the maximum yield of chlorophyll *a* fluorescence obtained after the addition of the PS II inhibitor, DCMU was compared to the

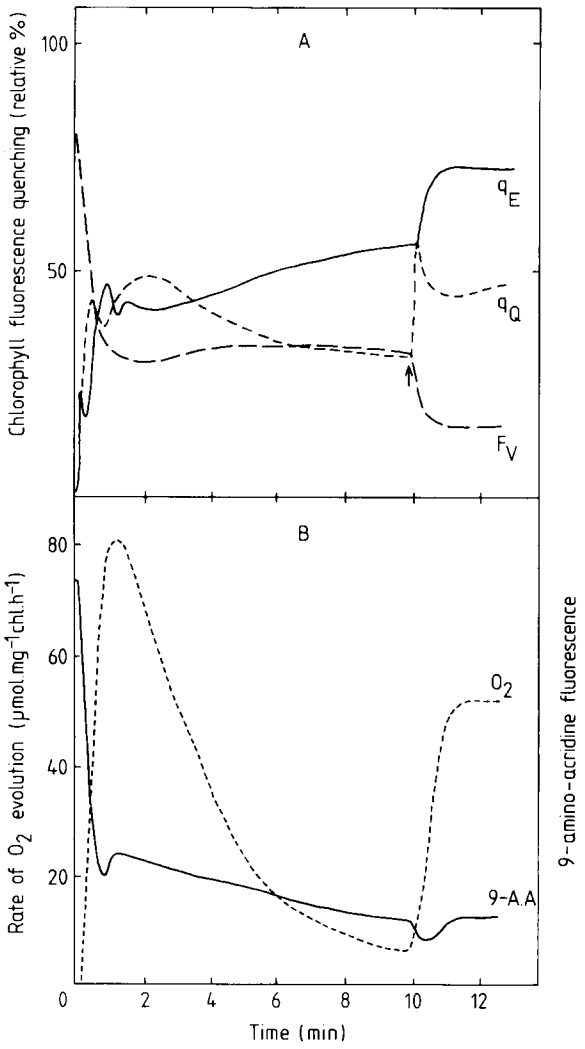

Fig. 5. The effect of P_i addition (0.1 mM) on CO_2 dependent O_2 evolution (·····), q_Q (— — —), q_E (———) and 9-aminoacridine fluorescence (— — —) in isolated chloroplasts after illumination in the absence of added P_i.

TABLE I

LOSS OF PHOTOSYNTHETIC CAPACITY AND VARIABLE CHLOROPHYLL FLUORESCENCE AFTER ILLUMINATION OF CHLOROPLASTS IN THE ABSENCE OF INORGANIC PHOSPHATE

Illumination period without P_i (min)	O_2 evolution after P_i addition (μmol per mg Chl per h)	F_{DCMU}/F_m
0	89	0.91
2	108	0.9
4	82	0.9
6	72	0.88
8	62	0.86
10	44	0.86

maximum fluorescence obtained on illumination from darkness with a saturating flash (F_m). This provides a measure of the loss of variable fluorescence in these experiments [27]. Chlorophyll fluorescence in the presence of DCMU decreased to 86% of the initial value of F_m after 10 min illumination, indicating negligible photoinhibitory damage to Photosystem II. These data also justifies the assumption that the most non-photochemical quenching of fluorescence, under these experimental conditions, resulted from high-energy state quenching and not photoinhibition.

Relief of P_i limitation in isolated chloroplasts and thylakoids

The cessation of O_2 evolution after P_i exhaustion could be caused by low P_i restricting dissipation of ΔpH through the ATP synthase or by diminished availability of NADP as an electron acceptor. The increase in q_E and 9-aminoacridine fluorescence quenching observed after P_i addition to illuminated chloroplasts (Fig. 5) suggests that the addition of P_i does not reduce the transthylakoid ΔpH as might be expected if P_i limitation of the thylakoid ATPsynthase was the major constraint. Fig. 6 illustrates this effect when methyl viologen was used as the electron acceptor in experiments with thylakoids. As P_i was consumed, q_E and 9-aminoacridine fluorescence increased but in this case, subsequent addition of 1.0 mM P_i then caused the decrease in both q_E and 9-aminoacridine fluorescence which would be expected in these circumstances. This is clearly consistent with a P_i limitation of the ATP-synthase increasing the transthylakoid ΔpH.

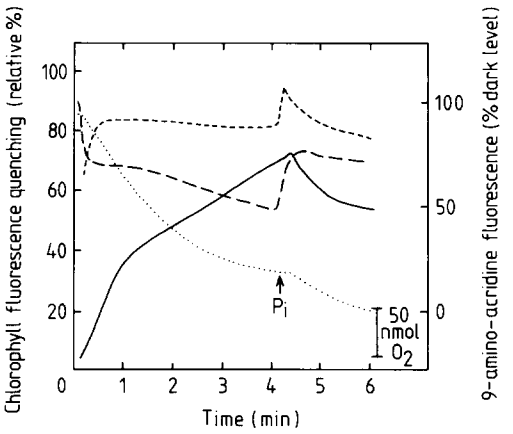

Fig. 6. Oxygen consumption (·····), q_Q (— — —), q_E (———) and 9-aminoacridine fluorescence (— — —) in osmotically shocked chloroplasts illuminated in the presence of methyl viologen (0.1 mM) in the presence of 1 mM ADP but in the absence of added P_i. P_i (1 mM) was added at the point indicated.

Discussion

The measurements of chloroplast [P_i] show that, in P_i-limited chloroplasts in which photosynthesis was completely inhibited, approx. 2 mM P_i is still present in the stroma. It appears that much of this pool is not readily available for photophosphorylation. Recent evidence suggests that a large fraction of stromal P_i may be bound to ribulose 1,5-bisphosphate carboxylase [15,16]. The rate of ATP synthesis may therefore be affected by the rate of binding and release of P_i from stromal proteins. If this fraction of bound P_i were assumed to be unavailable for photosynthesis under all conditions, then the 'free' P_i content of the stroma of illuminated chloroplasts supplied with optimal phosphate would be overestimated by at least 25%.

The data presented here show that P_i limitation of photosynthesis affects both carbon metabolism and electron transport. As a result of P_i exhaustion, ΔpH and q_E increase while the ATP/ADP ratio, q_Q and O_2 evolution simultaneously decrease. These events are in accord with photosynthetic control of electron transport or inhibition of

glycerate 3-phosphate kinase by ADP [28,29], or both. The fact that the concentration of glycerate 3-phosphate is as high in P_i-deficient chloroplasts as it is in chloroplasts supplied with P_i indicates that glycerate 3-phosphate consumption is inhibited. This is consistent with early observations [2] that glycerate 3-phosphate alone will not restore O_2 evolution in P_i-deficient chloroplasts, whereas glycerate 3-phosphate plus a small amount of P_i will. The triose phosphate concentration did not fall as a result of P_i-deficiency indicating that there was an additional limitation in triose phosphate consumption. The increased ribulose-1,5-bisphosphate concentration in P_i-limited chloroplasts clearly points to inhibition of ribulose-1,5-bisphosphate carboxylase activity. The fact that the glycerate 3-phosphate/triose phosphate ratio did not re-adjust according to the requirements of mass action is also in accord with additional inhibition within the Calvin cycle. Similarly, it is clear that the addition of P_i restores non-cyclic electron transport. However, the fluorescence changes occurring after the addition of P_i cannot be used to distinguish between a release of a block on the Calvin cycle or a release of the inhibition on photophosphorylation. Similarly, the changes in metabolite levels following P_i addition to P_i-depleted chloroplasts reported by Giersch and Robinson [31] do not clarify the situation or resolve the sequence of events at the sites of control in these circumstances. The proton gradient which has increased during P_i exhaustion (presumably because of pseudocyclic and cyclic electron transport) is abruptly increased on P_i addition, indicating that the sudden increase in non-cyclic electron flow has more than compensated for any discharge of the proton gradient through the ATP-synthase. The responses upon addition of nigericin are consistent with this hypothesis; however, the response upon addition of methyl viologen is more difficult to interpret. In this respect the behaviour of the P_i-deficient chloroplasts supplied with CO_2 differs from that of the thylakoids reducing methyl viologen in the absence of P_i. The latter displays changes in q_E and 9-aminoacridine fluorescence consistent with a discharge of the proton gradient after P_i addition. The difference lies in the fact that in P_i-deficient intact chloroplasts non-cyclic electron transport is co-limited by the availability of electron acceptor (NADP) and by ATP turnover and that in the intact system, these factors are inextricably linked. With methyl viologen the rate of noncyclic electron flow is increased most probably at the expense of cyclic electron flow. In the absence of methyl viologen intact chloroplasts will support a rate of noncyclic electron flow compatible with the rate of NADPH reoxidation by the NADP-glyceraldehyde 3-phosphate dehydrogenase reaction. The rate of noncyclic electron transport supported by this reaction will be dependent on the rate of glycerate-1,3-bisphosphate production by the phosphoglycerate kinase reaction which is determined primarily by the concentrations of ATP and glycerate 3-phosphate. Glycerate 3-phosphate will, therefore, reverse the inhibition of CO_2-dependent O_2-evolution by super-optimal P_i [2]. When glycerate 3-phosphate is added to chloroplasts inhibitedby high P_i, ΔpH and q_E are seen to increase, presumably because of the increased rate of NADP regeneration provided by the added substrate (Walker, D.A., unpublished observations). For this regulatory mechanism to occur in vivo, the pyridine nucleotide pool would have to be quite reduced, contrary to NADPH/NADP ratios measured in isolated chloroplasts which rarely exceed 3 and are more commonly below 2 [30]. The reliability of metabolite measurements as indicators of photosynthetically active pools is, in this case, doubtful due to extensive binding of pyridine nucleotides to ribulose-1,5-bis-phosphate carboxylase [14]. However, if Photosystem I was highly reduced one would expect Q_A to become highly reduced. From Figs. 1 and 5 this does not appear to be the case.

The large increase in ribulose 1,5-bisphosphate concentration observed on illumination of P_i-deficient chloroplasts implies that ribulose 1,5-bisphosphate carboxylase activity is severely inhibited by low P_i. However, other observations have shown [31] that although P_i exhaustion diminished the activation stage of ribulose 1,5-bisphosphate carboxylase, it did not decrease it to a level that would significantly restrict flux through the Calvin cycle. Orthophosphate has previously been found to be required for activation of ribulose 1,5-bisphosphate carboxylase in isolated chloroplasts [18] and deactivation of the enzyme has also been observed in leaves under conditions in

which P_i is believed to limit photosynthesis in vivo [32]. No changes in the activation state could be detected in the present experiments during P_i exhaustion. Both control and 'low P_i' chloroplasts contained fully activated carboxylase in the light and dark. It appears therefore that inhibition of catalysis rather than a decline in the activation state is responsible for the accumulation of ribulose 1,5-bisphosphate under P_i limitation. On the basis of the metabolite data alone it is not possible to determine the basis of this regulation. Glycerate 3-phosphate does not accumulate to a concentration which would significantly inhibit catalysis by competitive inhibition of ribulose 1,5-bisphosphate binding site [33,34].

Illumination of chloroplasts in the absence of added P_i also caused an accumulation of fructose 1,6-bisphosphate. This has been seen by other workers [31] and suggests that there may also be an inhibition of fructose 1,6-bisphosphatase activity in these circumstances. Both ribulose 1,5-bisphosphate carboxylase and fructose 1,6-bisphosphatase are inhibited by high [P_i] and would be expected to be most active when stromal [P_i] was low [34–36]. In these experiments the converse effect is indicated.

Chloroplasts illuminated in the presence of CO_2 but without added P_i showed a progressive loss of photosynthetic capacity [Table I]. Such light-dependent loss of photosynthetic activity is termed photoinhibition and has often been attributed to damage of electron transport in Photosystem II [27]. However, in these experiments no appreciable loss of variable fluorescence was observed and the major component of non-photochemical quenching appeared to be high-energy-state quenching (q_E). The light intensities used in these experiments were 70–80% saturating which may account for lack of 'classical' photoinhibition observed in other studies at higher light intensities. However, these results indicate that light-dependent damage to the photosynthetic apparatus other than damage to Photosystem II may occur at moderate light intensities in the absence of P_i.

It is evident that there is a complex relationship between q_Q and the rate of O_2 evolution during the induction phase of photosynthesis. Following illumination Q_A was rapidly oxidised without concomitant net O_2 evolution. If it is assumed that the redox state of Q_A is dependent on the rate of non-cyclic electron transport, then substantial electron transport to an acceptor other than NADP must be occurring. It has previously been shown that the rate of pseudocyclic electron transport in chloroplasts is higher during the induction phase of CO_2-dependent O_2 evolution [37]. Similarly, after net O_2 evolution ceased in P_i-depleted chloroplasts an appreciable amount of photochemical quenching remained, suggesting that electron transport was occurring which did not result in a change in O_2 concentration. Either pseudocyclic or cyclic electron transport might affect the oxidation state of Q_A and operation of the pathways might be responsible for this residual q_Q. It has also been suggested that the redox state of Q_A may not always reflect the rate of CO_2 fixation in intact leaves due to a feed-back regulation of Photosystem II by the ΔpH [38]. These authors propose that at high levels of non-photochemical quenching the quantum yield of open reaction centres in Photosystem II is lowered, limiting the rate of electron donation to Q_A thus keeping Q_A more oxidised than expected. For this mechanism to regulate electron transport during P_i limitation, it would have to respond to both ΔpH and redox state, since differences in ΔpH between P_i treatments were not large.

In conclusion, photosynthesis under P_i-limiting conditions is regulated both at the thylakoid level and by the enzymes of carbon metabolism in a concerted fashion. There clearly appears to be a complex series of interactions between the thylakoid and stromal reactions which provides an efficient synchronous regulation of photosynthetic flux.

Acknowledgements

The authors wish to thank Simon Robinson, Peter Horton, Don Ort, Ulrich Heber, Richard Leegood and Mirta Sivak for stimulating discussion of these results. This work was supported by the Agricultural and Food Research and Science and Engineering Research Councils of the United Kingdom.

References

1 Cockburn, W., Baldry, C.W. and Walker, D.A. (1967) Biochim. Biophys. Acta 131, 594–596
2 Cockburn, W., Baldry, C.W. and Walker, D.A. (1967) Biochim. Biophys. Acta 143, 614–624
3 Walker, D.A. and Crofts, A.R. (1970) Annu. Rev. Biochem. 39, 389–428
4 Heldt, H.W. and Rapley, L. (1970) FEBS Lett. 10, 143–148
5 Walker, D.A. and Herold, A.R. (1977) in Photosynthetic Organelles: Structure and Function (Fujita, Y., Fatoh, S., Shibata, K. and Miyachi, S., eds.), pp. 295–310, Japanese Society of Plant Physiologists and Centre for Academic Publications, Tokyo
6 Walker, D.A. and Robinson, S.P. (1978) Ber. Deutsch. Bot. Ges. 91, 513–526
7 Walker, D.A. and Sivak, M.N. (1986) Trends Biochem. Sci. 11, 176–179
8 Sheu-Hua, C.-S., Lewis, D.H. and Walker, D.A. (1975) New Phytol. 74, 383–392
9 Foyer, C.H. and Spencer, C. (1986) Planta 167, 369–375
10 Laisk, A. and Walker, D.A. (1986) Proc. R. Soc. Lond. B 227, 281–302
11 Sivak, M.N. and Walker, D.A. (1987) Plant Physiol. Biochem., in press
12 Sivak, M.N. (1987) Photobiochem. Photobiophys., in press
13 Selman, B.R.and Selman-Reimer, S. (1981) J. Biol. Chem. 256, 1722–1726
14 Ashton, A.R. (1982) FEBS Lett. 145, 1–6
15 Furbank, R.T., Foyer, C.H. and Walker, D.A. (1986) Biochim. Biophys. Acta 852, 46–54
16 Furbank, R.T., Foyer, C.H. and Walker, D.A. (1987) Biochim. Biophys. Acta 894, 165–173
17 Robinson, S.P. and Giersch, C. (1987) Aust. J. Plant Physiol., in press
18 Heldt, H.W., Chon, C.J. and Lorimer, G.H. (1978) FEBS Lett. 92, 234–240
19 Parry, M.A.J., Schmidt, C.N.G., Cornelius, M.J., Keys, A.J., Millard, B.N. and Gutteridge, S. (1985) J. Expt. Bot. 36, 1396–1404
20 Walker, D.A. (1987) in The Use of Oxygen Electrode and Fluorescence Probes in Simple Measurements of Photosynthesis, pp. 1–145, Oxygraphics Ltd. Sheffield
21 Quick, W.P. and Horton, P. (1984) Proc. R. Soc. Lond. B220, 361–370
22 Horton, P. (1983) Proc. R. Soc. Lond. B217, 405–416
23 Schreiber, U., Schliwa, W. and Bilger, U. (1986) Photosynthesis Res. 10, 51–62
24 Robinson, S.P. and Walker, D.A. (1979) Arch. Biochem. Biophys. 196, 319–323
25 Furbank, R.T. and Foyer, C.H. (1986) Arch. Biochem. Biophys. 246, 240–244
26 Itaya, K. and Ui, M. (1966) Clin. Chim. Acta 14, 361–366
27 Satoh, K. and Fork, D.C. (1982) Plant Physiol. 70, 1004–1008
28 Robinson, S.P. and Walker, D.A. (1979) Biochim. Biophys. Acta 545, 528–536
29 Pacold, I. and Anderson, L.E. (1975) Plant Physiol. 55, 168–171
30 Takahama, U., Shimizu-Takahama and Heber, U. (1981) Biochim. Biophys. Acta 637, 530–539
31 Giersch, C. and Robinson, S.P. (1987) Planta, in press
32 Sharkey, T.D., Seemann, J.R. and Berry, J.A. (1986) Plant Physiol. 81, 788–791
33 Robinson, S.P., McNeil, P.H. and Walker, D.A. (1979) FEBS Lett. 97, 296–300
34 Badger, M.R. and Lorimer, G.H. (1981) Biochemistry 20, 2219–2225
35 Foyer, C.H., Furbank, R.T. and Walker, D.A. (1987) Biochim. Biophys. Acta 894, 157–164
36 Furbank, R.T. and Lilley, R.McC. (1980) Biochim. Biohyps. Acta 592, 65–75
37 Furbank, R.T., Badger, M.R. and Osmond, C.B. (1982) Plant Physiol. 70, 927–931
38 Weis, E., Berry, J. and Ball, T. (1986) in Progress in Photosynthesis Research (Biggins, J., ed.), Vol. II, pp. 553–586, Martinus Nijhoff, Dordrecht