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ENERGETICS OF PHOTOSYNTHESIS IN *ZEA MAYS*

II. STUDIES OF THE FLASH-INDUCED ELECTROCHROMIC SHIFT AND FLUORESCENCE INDUCTION IN MESOPHYLL CHLOROPLASTS

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Patterns of electron transfer in isolated mesophyll chloroplasts of maize (*Zea mays* L.) were studied in the presence of the physiological substrates, oxaloacetate, 3-phosphoglycerate and pyruvate. Flash-induced absorbance changes due to the electrochromic pigment band-shift (P-518) were used to estimate relative electron flow rates through the cyclic and non-cyclic pathways of electron transport. Further information on the redox state of electron carriers and the activity of coupled electron flow was obtained from measurements of fluorescence induction and of actinic-light-induced fluorescence changes. The results demonstrate the importance of correct redox poising for optimal rates of photosynthesis and are discussed in relation to the operation and regulation of photosynthesis in the C_4 system.

Introduction

The pathway of photosynthetic carbon assimilation in C_4 plants such as maize has been elucidated through studies both of enzyme localisation [1–4] and of the photosynthetic capacities of isolated mesophyll protoplasts and chloroplasts and bundle sheath strands [5–8]. The cytoplasm of the mesophyll cells is the site of carboxylation of phospho*enol*pyruvate and the resulting oxaloacetate is reduced to malate in the mesophyll chloroplasts. Malate is then transferred to the bundle sheath where it is decarboxylated to yield CO_2, NADPH and pyruvate. The pyruvate returns to the mesophyll chloroplast and is phosphorylated to regenerate phospho*enol*pyruvate, completing the cycle. Since the bundle sheath chloroplast is deficient in PS II activity [4,7–11] and malate decarboxylation generates only half of the NADPH requirement for 3-phosphoglycerate reduction, up to half of the 3-phosphoglycerate formed in the Benson-Calvin cycle must be exported from the bundle sheath to the mesophyll chloroplast for conversion to triose phosphate and then returned to the bundle sheath, forming a subsidiary cycle.

When the energetic requirements of this pathway are considered, it is evident that the fixation of one molecule of CO_2 into triose phosphate

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Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Mes, 2-(*N*-morpholino)ethanesulphonic acid; Tricine, *N*-tris(hydroxymethyl)methylglycine; F_0, intrinsic bed fluorescence; F_i, initial level of fluorescence seen during rapid induction (F_0 plus a variable component proportional to the state of reduction of Q); F_m, maximal level of fluorescence (seen in the presence of DCMU); F_v, variable fluorescence (maximally $F_m - F_0$); Chl, chlorophyll; PS, photosystem.

requires the generation of two ATP molecules in the bundle sheath chloroplast plus three ATP and two NADPH molecules in the mesophyll chloroplast. In the PS II-deficient bundle sheath, ATP-generating cyclic electron flow is therefore the principal mode of electron transport [8–10], whereas in the C_4 mesophyll the overall energetic requirement from the chloroplast is the same as in spinach. Although the spinach chloroplast displays considerable flexibility in the utilisation of linear and cyclic pathways, allowing ATP and NADPH to be produced in ratios appropriate to varying demands (for example, during the onset of CO_2 fixation) [12], in vitro such flexibility can be more obviously demonstrated in the C_4 mesophyll chloroplast, since the energetic demands placed upon it can be radically manipulated by the addition of different substrates. This is possible because the chloroplast contains high activities of enzymes catalysing the independent conversions of pyruvate to phospho*enol*pyruvate, oxaloacetate to malate and 3-phosphoglycerate to triose phosphate [1–4], requiring ATP alone, NADPH alone or ATP plus NADPH, respectively. For instance, cyclic electron flow has been demonstrated in mesophyll chloroplasts of *Digitaria sanguinalis* with pyruvate present (phospho*enol*poyruvate production being monitored by $^{14}CO_2$ fixation in the presence of phospho*enol*pyruvate carboxylase) [13–15]. Those studies, together with observations made with maize mesophyll chloroplasts [5], showed that cyclic photophosphorylation can proceed at appreciable rates, particularly if properly poised by the addition of low concentrations of DCMU or if chloroplasts are illuminated with far-red light.

This investigation, using absorbance and fluorescence spectroscopy to monitor electron flow, further demonstrates the flexibility in electron-flow patterns that may be observed in mesophyll chloroplasts, shows the central role of redox poise in this flexibility and discusses the advantages to the C_4 plant that may arise from it.

Materials and Methods

Materials. Zea mays (Pioneer Hybrid 3780) was grown during the summer in a naturally illuminated greenhouse. Cellulase was purchased from Yakult Biochemicals Ltd., Nishinomiya, Japan, and pectinase from Calbiochem. San Diego, CA. Nigericin was kindly given by Dr. R.L. Hamill of Eli Lilly Laboratories.

Preparation of mesophyll protoplasts and chloroplasts. For the isolation of protoplasts, leaf segments (0.5–1.0 mm wide) were cut by hand from 2-week-old leaves and incubated under light at 28°C in 50 ml of a medium containing 0.5 M sorbitol, 1 mM $CaCl_2$, 2% (w/v) cellulase (Onozuka 3S), 0.3% (w/v) pectinase (Macerase) and 5 mM Mes (pH 5.5). The leaf segments were incubated for 2 h and protoplasts collected and purified as described previously [16], except that the crude filtrate was also passed through a 71 μm mesh and the gradient for purification consisted of 5 ml of 0.5 M sucrose, 1 mM $CaCl_2$, 5 mM Mes (pH 6.0) on top of which was layered 1 ml of 0.5 M sorbitol, 1 mM $CaCl_2$, 10 mM Tricine (pH 7.6) (in which medium the protoplasts were also stored). For chloroplast preparation, small aliquots of protoplasts (about 400μg Chl) were diluted with 0.5 M sorbitol, pelleted by centrifugation at $100 \times g$ for 1 min and resuspended in 0.5 ml 0.4 M sorbitol, 10 mM EDTA, 25 mM Tricine (pH 8.0). Protoplasts were then broken by passing them three times through a 20-μm nylon mesh attached to a 1-ml plastic syringe. Chloroplasts were collected by centrifugation at $250 \times g$ for 45 s, and the pellet was resuspended in the same medium. The percentage of intact chloroplasts as determined by ferricyanide-dependent O_2 evolution before and after osmotic shock [17] was approx. 85% and the chloroplasts showed rates of 3-phosphoglycerate-dependent O_2 evolution in excess of 100 μmol/h per mg Chl at 20°C.

Spectrophotometry. Flash-induced absorbance changes were measured as previously described [10]. Each trace represents the average of 128 records.

Chlorophyll fluorescence. Chlorophyll fluorescence induction was measured as previously described [10]. Slow changes in fluorescence during strong blue actinic illumination (measuring beam intensity 0.1 $W \cdot m^{-2}$ root mean square; actinic beam intensity 25 $W \cdot m^{-2}$) were measured with a beam modulated at 270 Hz and a lock-in amplifier.

Anaerobic samples. Media were gassed with Ar.

Sample cuvettes were filled with N_2 and maintained in an anaerobic state by the addition of 10 mM glucose, 500 U/ml glucose oxidase and 1100 U/ml catalase.

Chlorophyll. Chlorophyll was determined by the method of Arnon [18].

Results

P-518 in mesophyll chloroplasts

The flash-induced electrochromic band-shift (P-518) was measured during trains of actinic flashes and the relative extents of the fast ($P\text{-}518_f$) and slow ($P\text{-}518_s$) phases used to indicate the activities of non-cyclic and cyclic electron flows. Non-cyclic electron flow to an acceptor gives rise to a large $P\text{-}518_f$, while cyclic electron flow gives rise to a smaller $P\text{-}518_f$ and large $P\text{-}518_s$ [10].

The P-518 observed in maize mesophyll chloroplasts was, on a chlorophyll basis, comparable in amplitude to that observed in spinach chloroplasts [19] and considerably larger than the signal measured in bundle sheath strands [9,10]. On addition of orthophosphate and catalase only, a pronounced $P\text{-}518_s$ was observed (Fig. 1) with a less than maximal $P\text{-}518_f$, showing some cyclic electron flow and an overall reduced state of intersystem electron carriers. Omission of catalase led to an inhibition of the rise time and amplitude of $P\text{-}518_s$ (results not shown) which may partly account for the slow rise time of $P\text{-}518_s$ (approx. 40 ms) previously observed in maize mesophyll chloroplasts [20,21]. When pyruvate was added (which causes ATP consumption through its conversion to phospho*enol*pyruvate catalysed by pyruvate, orthophosphate dikinase) the rise rate and amplitude of $P\text{-}518_s$ were increased, showing a decrease in the transthylakoid ΔpH caused by ATP consumption. The $P\text{-}518_s$ extent and rise rate were further increased by the addition of nigericin, as would be expected if ΔpH were restricting cyclic electron flow [22]. $P\text{-}518_s$ was lost and $P\text{-}518_f$ increased upon addition of oxaloacetate, showing a change to linear electron flow to oxaloacetate and an oxidation of intersystem electron carriers; oxaloacetate is an efficient electron acceptor in maize mesophyll chloroplasts because of the presence of high activities of NADP-dependent malate dehydrogenase. In spinach chloroplasts, where only a low activity of this enzyme is usually present [23], oxaloacetate scarcely affects P-518 (Crowther, D. and Hind, G., unpublished results). If DCMU was added subsequent to oxaloacetate, an almost total inhibition of P-518 resulted, presumably because the electron sink provided by oxaloacetate is so effective that PS I electron donors are not re-reduced between the (2 Hz) flashes (this observation may be compared with the effect that an efficient electron acceptor such as methyl viologen has on the P-518 in PS II-deficient bundle sheath strands [10]). If DCMU was added to mesophyll chloroplasts in the absence of oxaloacetate or any other added electron acceptor, then $P\text{-}518_f$ was reduced by only approx. 50% at this flash frequency (results not shown).

Addition of malate subsequent to DCMU failed to restore P-518, in contrast to its effect in maize bundle sheath strands [10]. This is probably due to

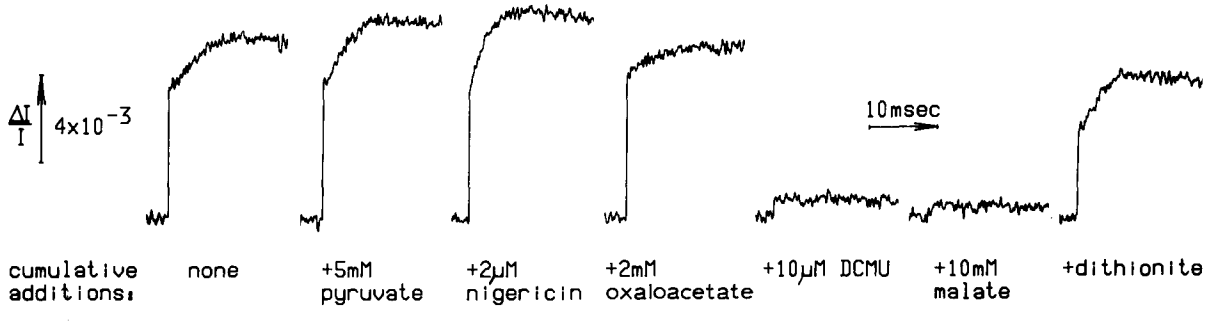

Fig. 1. Flash-induced absorbance change at 518 nm in maize mesophyll chloroplasts. The reaction mixture (1.5 ml) contained 0.4 M sorbitol, 10 mM EDTA, 25 mM Tricine (pH 8.0) and chloroplasts equivalent to 105 μg Chl. Sequential additions to the same sample were made as shown.

lack of NADPH production, since mesophyll chloroplasts lack NADP-malic enzyme and the equilibrium of the reaction catalysed by malate dehydrogenase lies strongly in favour of malate formation [24], whereas in the bundle sheath decarboxylation of malate by NADP-malic enzyme yields NADPH which can in turn donate electrons to the cyclic pathway. However, dihydroxyacetone phosphate, the addition of which to chloroplasts can lead to NADPH generation by a different pathway [25], was also ineffective in poising cyclic electron flow under either aerobic or anaerobic conditions (results not shown). After DCMU addition an appropriate redox poise to support cycling, demonstrated by a restored P-518$_s$, could be obtained by adding dithionite [22]; this suggests that a limiting supply of reductant was the reason for the above effects.

Fig. 2 shows the effects of oxaloacetate and of anaerobiosis upon P-518 in mesophyll chloroplasts. In the aerobic sample, addition of nigericin decreased the rise time of P-518$_s$ and subsequent addition of oxaloacetate both increased P-518$_f$ and removed the slow phase (as in Fig. 1). In this respect, the addition of oxaloacetate also resembles the addition of nitrite to maize mesophyll chloroplasts (Fig. 3) or of methyl viologen to spinach chloroplasts [26]. In each case, the stimulation of P-518$_f$ and the removal of P-518$_s$ result from an increase in the rate of linear electron flow at the expense of electrons from the cyclic pathway. Anaerobiosis in the presence of only orthophosphate and catalase (Fig. 2) led to a stimulation of P-518$_s$, but when the chloroplasts were

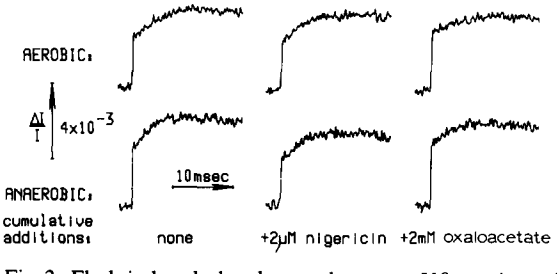

Fig. 2. Flash-induced absorbance change at 518 nm in maize mesophyll chloroplasts. Reaction conditions were as described in the legend to Fig. 1, except that the chlorophyll concentration was 67 μg Chl in 1.5 ml. Reaction mixtures were made anaerobic as described in Materials and Methods (lower series of traces). Sequential additions were made as shown.

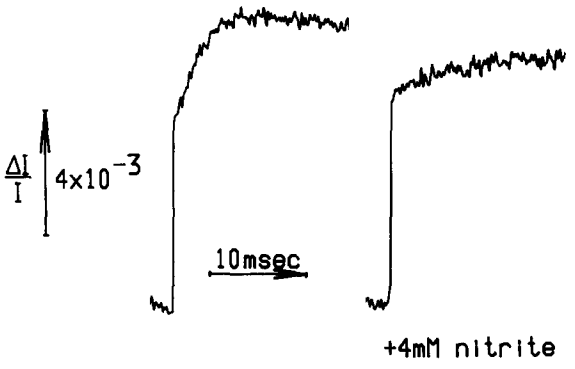

Fig. 3. Influence of nitrite on the flash-induced absorbance change at 518 nm in mesophyll chloroplasts. Reaction conditions were as described in the legend to Fig. 1 except that the initial reaction medium was supplemented with 5 mM pyruvate and 2 μM nigericin. The chlorophyll concentration was 105 μg Chl in 1.5 ml. Nitrite was added following the first measurements.

uncoupled by the addition of nigericin there was a decrease in the amplitude of both phases of P-518 and a slowing of the rise time of P-518$_s$; this is consistent with anaerobic conditions promoting an over-reduction of the mesophyll electron-transport chain, and aggravation of this by uncoupler-stimulated electron input. An electron acceptor, oxaloacetate, readily relieved this, resulting in an increase in P-518$_f$ and a diminished P-518$_s$, although inhibition of P-518$_s$ was slight compared with that observed under aerobic conditions.

Fig. 3 illustrates the effect of nitrite on P-518 in aerobic maize mesophyll chloroplasts (the principal site of nitrite reduction in the maize leaf [27]). Like oxaloacetate (Fig. 2), it removed P-518$_s$ and promoted P-518$_f$ by acting as an electron sink.

Chlorophyll fluorescence induction

When leaves, protoplasts or chloroplasts are illuminated, they show characteristic changes in Chl *a* fluorescence with both fast phases (which occur within a few seconds) and much slower phases (which may persist for several minutes) [28]. The fast phases seen in fluorescence induction curves indicate the initial redox state of PS II acceptors shown by F_i, the initial fluorescence, and the rates of electron flow into and out of the plastoquinone pool which are shown by the subse-

quent induction curve. On actinic illumination, following the rise of fluorescence to a peak, a slow quenching occurs as photosynthesis reaches a steady-state rate. Investigation of this slow fluorescence quenching has shown it to be a complex process determined by several factors, including the redox state of Q (and thus, indirectly, the redox state of the pyridine nucleotide pool [29]), the energy status of the thylakoid membrane (principally ΔpH) and the phosphorylation status of membrane proteins (part of the light-harvesting complex of PS II, affecting energy transfer) [29–33].

Fast and slow changes in chlorophyll fluorescence induction were measured in maize mesophyll chloroplasts which had been isolated in the dark from dark-adapated protoplasts. Fig. 4 shows the fluorescence induction curves obtained over 10 s of weak illumination. These curves were sigmoidal (as typically seen in C_3 species [28]) and the ratio of variable fluorescence to F_i was about 5, similar to the ratio found in spinach [34]. When compared with bundle sheath strands, the extent of fluorescence was much greater on a chlorophyll basis [10,11], indicating that fluororescence measured in leaves would arise almost wholly from mesophyll cells. Added substrates elicited quite different induction curves from those obtained in the bundle sheath [10]. In the presence of pyruvate there was a rapid increase in fluorescence from F_i to the first plateau and dip in fluorescence (I-D in the nomenclature of Munday and Govindjee [35]), followed by a rapid increase to the fluorescence peak, which approached the maximal fluorescence intensity, F_m, observed in the presence of DCMU. In the presence of 3-phosphoglycerate the rise to I-D was very slightly slower; the peak fluorescence intensity was lower and was followed by a steeper decline. Peak fluorescence was still less intense in the presence of oxaloacetate although on the first illumination the fluorescence decline was not as rapid as with 3-phosphoglycerate. If no additions other than orthophosphate and catalase were made the fluorescence induction curve fell between the 3-phosphoglycerate and pyruvate induction curves. Under anaerobic conditions in the presence of pyruvate, the same chloroplasts showed a rapid fluorescence rise to give a spike (sometimes more pronounced on second or third illuminations), followed by a more rapid rise to the peak than was observed in chloroplasts incubated with pyruvate alone.

Fig. 5 illustrates slow chlorophyll fluorescence quenching on actinic illumination observed under the same conditions as the fast fluorescence induction traces. Traces (not shown) obtained on subsequent illuminations of the same samples showed effects similar to, but generally more pronounced than those described here (i.e., more rapid and more extensive quenching in the presence of electron acceptors). In the presence of pyruvate under either aerobic or anaerobic conditions there was a rapid increase in fluorescence on actinic illumination followed by little or, frequently, no fluores-

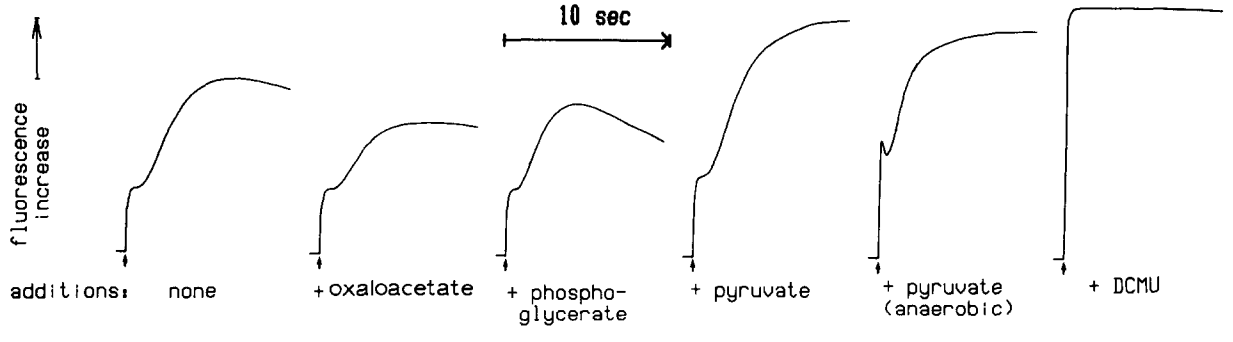

Fig. 4. Induction of chlorophyll fluorescence in a suspension of maize mesophyll chloroplasts. Fluorescence was excited and measured as described in Materials and Methods. The reaction mixture (3 ml) contained 0.4 M sorbitol, 10 mM EDTA, 25 mM Tricine (pH 8.0) and chloroplasts equivalent to 11 μg Chl. The traces were obtained from separately treated samples of dark-adapted chloroplasts. Concentrations of the additions were: oxaloacetate, 2 mM; 3-phosphoglycerate, 2 mM; pyruvate, 5 mM; DCMU, 10 μM. Samples were made anaerobic as described in Materials and Methods.

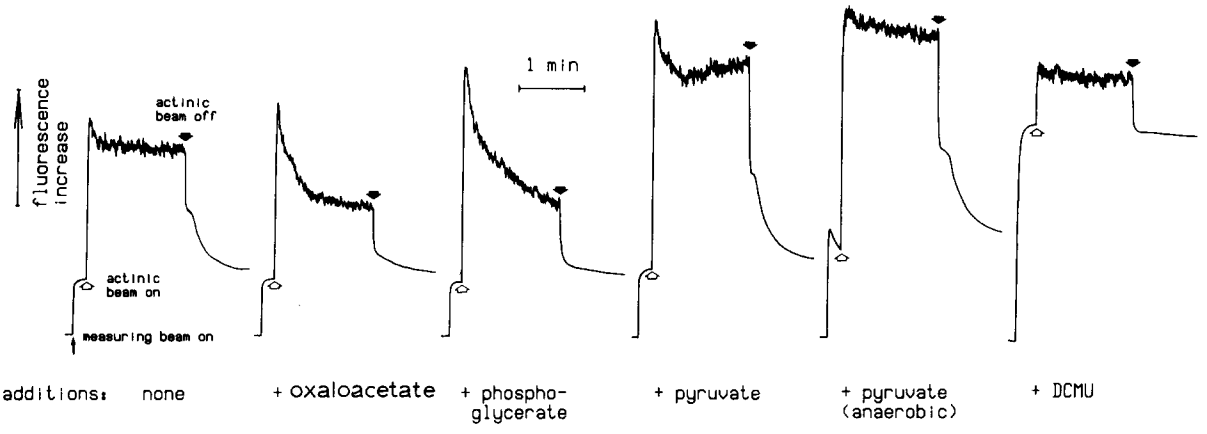

Fig. 5. Chlorophyll fluorescence yield in a suspension of maize mesophyll chloroplasts during actinic illumination. Fluorescence was excited and measured as described in Materials and Methods. Reaction conditions and concentrations of additions were as described in the legend to Fig. 4 except that the chlorophyll concentration was 11 μg Chl in 3 ml. Reaction mixtures were made anaerobic as described in Materials and Methods. The traces were obtained from separately treated samples of dark-adapted chloroplasts.

cence quenching. When actinic illumination was switched off there was a triphasic recovery; an initial fast fluorescence decline was followed by a plateau or even a slight increase in fluorescence, followed by a slow decline to the initial fluorescence intensity. This complex recovery was not observed in the presence of any other substrate tested. During actinic illumination with 3-phosphoglycerate as substrate, there was a rapid and extensive fluorescence quenching following the peak (this quenching was more rapid on subsequent illuminations) and on switching off the light there was a slow decline to the initial level of fluorescence. Addition of oxaloacetate also resulted in extensive slow fluorescence quenching, although in this case the transient peak in fluorescence intensity was much lower than that from 3-phosphoglycerate and pyruvate additions, especially on second and subsequent illuminations, which may reflect a requirement for light-dependent reductive activation of NADP-malate dehydrogenase [24,36]. Inclusion of DCMU resulted in a high fluorescence intensity driven by the measuring beam and an additional increment elicited by actinic illumination.

Discussion

The reactions of the C_4 pathway which are mediated by the maize mesophyll chloroplast (conversion of pyruvate, 3-phosphoglycerate and oxaloacetate) require the steady-state generation of three ATP and two NADPH molecules per CO_2 molecule fixed. As linear electron flow is likely to result in a shortfall of ATP, the balance of the ATP requirement must be met by cyclic photophosphorylation or by electron flow to O_2 [12,37]. In spinach chloroplasts, appropriate ratios of ATP to NADPH may be generated by flexible routing of electrons through the cyclic and non-cyclic pathways (with only a small contribution from the Mehler reaction) [12,38]. In maize mesophyll chloroplasts, the reactions of the C_4 pathway should proceed at approximately equal rates during steady-state CO_2 fixation, but during photosynthetic induction these would probably vary in relation to one another, placing different energetic demands upon the chloroplast; this would necessitate a flexibility in ATP and NADPH production at least as great as that seen in spinach chloroplasts.

Cyclic electron flow is very sensitive to redox poise and is delicately balanced with linear electron flow in chloroplasts with substantial PS II content. Inhibition of cyclic electron flow readily results from over-reduction by electrons from PS II, particularly at high light intensities. Over-reduction can be relieved by such treatments as partial inhibition of PS II input by DCMU [39,40], by additional far-red light [14,41] or, under ap-

propriate conditions, by electron acceptors [39,40,42]. Conversely, if the input of electrons from PS II is weak [9,10] or is blocked by DCMU, over-oxidation ensues, a situation which is exacerbated by electron acceptors [10]. In vivo, increasing concentrations of electron acceptors would lead to a progressive loss of cyclic electron flow and an increase in linear electron flow from water to the acceptor.

The results presented here demonstrate that in weakly illuminated aerobic maize mesophyll chloroplasts, addition of pyruvate enhances a rate of cyclic electron flow already pronounced in the absence of substrate. The P-518 response in the presence of pyruvate (Fig. 1) indicates rapid turnover of cyclic electron flow and the fluorescence induction curve (Fig. 4) shows a rapid increase to a reduced state at the onset of illumination, as would be expected if pyruvate acts as an ATP sink allowing rapid electron flow to reduce the small endogenous pools of electron acceptors. Under strong actinic illumination an over-reduction would be expected; this may account for the phenomenon observed in Fig. 5 where, in the presence of pyruvate, transient fluorescence quenching was observed on actinic illumination, but was then lost and the fluorescence rose to a near-maximal level (i.e., this transient quenching may represent an initial energy-dependent quenching of fluorescence). Under flash (weak) illumination and in the presence of pyruvate as an ATP sink, nigericin relieved the constraint of ΔpH on cyclic electron flow (Fig. 1) and a large P-518$_s$ and smaller P-518$_f$ were seen; subsequent addition of oxaloacetate (an electron acceptor not requiring ATP) restored P-518$_f$ to a near-maximal level and decreased the extent of P-518$_s$, showing a switch to predominantly non-cyclic electron flow. Subsequent addition of DCMU resulted in an over-oxidation of intersystem carriers (little re-reduction of PS I between flashes and therefore a minimal P-518), following which cyclic activity could be restored by redox poising with dithionite, as seen in spinach chloroplasts [19,22].

Comparison of the effects of electron acceptors on the P-518 change under both aerobic and anaerobic conditions (Fig. 2) illustrates that, in weak light, anaerobiosis can lead to an enhancement of cyclic electron flow (the extent of P-518$_s$ relative to that of P-518$_f$ being greater in anaerobic samples), showing that oxygen inhibits cyclic electron flow when overall electron flow rates (governed principally by ΔpH) are low [38,41]. However, under uncoupled conditions anaerobiosis led to a reduction in the rate of cyclic flow (a smaller P-518$_s$; Fig. 2, lower centre), indicating over-reduction when electron flow could proceed rapidly. Anaerobiosis also led to rapid reduction of PS II acceptors on illumination in the presence of pyruvate as an ATP sink (Fig. 4), the fluorescence induction curve rising rapidly to near the maximal level. Fluorescence induction in an aerobic sample with pyruvate present was slower but still reached a high level. The initial level of fluorescence in the aerobic sample was considerably lower than that in the anaerobic sample, however, suggesting that some PS II acceptors were reduced in the dark in the anaerobic sample but not in the aerobic sample. The appearance of a fluorescence spike under anaerobiosis is reminiscent of similar observations made on anaerobic *Chlorella* cells [28]. With actinic illumination (Fig. 5) no slow fluorescence quenching was seen in the anaerobic sample with pyruvate, suggesting a rapid over-reduction of the electron-transfer chain. The fluorescence induction curve (Fig. 4) measured with oxaloacetate present exhibited a lower final level of fluorescence, as would be expected from its ability to act solely as an oxidant of NADPH, while 3-phosphoglycerate (requiring both ATP and NADPH for its conversion to triose phosphate) caused the fluorescence induction curve to rise and then fall again, a feature which suggests a lag in 3-phosphoglycerate reduction during which ATP levels are built up, partly by cyclic electron flow [43]. Under strong illumination (Fig. 5), traces obtained in the presence of oxaloacetate and of 3-phosphoglycerate showed considerable slow fluorescence quenching. Krause et al. [29] have shown energy-dependent quenching to be high under conditions of coupled electron flow to an electron acceptor (for example, in the presence of oxaloacetate). In this case, quenching could be reinforced both by the high energy state (ΔpH) of the thylakoid membrane and by the relatively oxidised state of Q.

Previous studies of chloroplasts from both maize and *D. sanguinalis* [5,13–15] also indicated balanc-

ing of electron flow rates by redox poise. In white light (where high PS II activity might be expected), low rates of phospho*enol*pyruvate formation from pyruvate were stimulated considerably by addition of electron acceptors such as methyl viologen [5], oxaloacetate [14] or oxygen [44]. These results would be expected if, with high PS II activity and with pyruvate as an ATP sink, over-reduction occurred under strong illumination with a consequent inhibition of ATP production mediated by cyclic electron flow; adjustment of redox poise with added electron acceptors could then allow ATP production to recommence. At low oxygen tensions, far-red illumination or low concentrations of DCMU greatly stimulated phospho*enol*-pyruvate formation and inhibition by oxaloacetate was then possible [13,14], further demonstrating the need for balanced input and output of electrons to allow ATP production to proceed optimally.

Although such over-reduction under anaerobiosis may be observed in maize mesophyll chloroplasts, studies with maize and *Amaranthus* leaves suggest that in C_4 plants this phenomenon is not as serious an impediment to CO_2 fixation as it is in C_3 plants [45,46]. This may be because the bundle sheath does not exhibit over-reduction phenomena [10] and because in vivo there is always an excess of electron acceptors which can overcome it [38]. Thus, the generation of oxaloacetate in the mesophyll cytoplasm and the 3-phosphoglycerate/dihydroxyacetone phosphate shuttle between the bundle sheath and mesophyll chloroplasts probably provides a more effective redox buffer capacity than is found in C3 chloroplasts, in which the buffer capacity is largely limited to the pyridine nucleotide pool. Comparison of the slow chlorophyll fluorescence quenching traces from maize mesophyll chloroplasts with data from spinach chloroplasts [29–31,39] shows that, in spinach, physiological substrates (3-phosphoglycerate, oxaloacetate and CO_2) have a much smaller effect on slow fluorescence quenching (and hence electron flow rates) than in C_4 species, reflecting the high activities of enzymes catalysing the independent conversions of substrates in the C_4 plant (in spinach chloroplasts, only linear electron flow to an artificial acceptor (such as methyl viologen) may be capable of matching or exceeding the rate of oxaloacetate-dependent linear electron flow seen in maize mesophyll chloroplasts). The C_4 system may therefore provide a less easily perturbed means of achieving the sensitive balance between linear and cyclic electron transport that is necessary for very high rates of CO_2 fixation.

Appendix

Estimation of relative linear and cyclic electron flow rates from P-518 changes

PS I and PS II produce P-518$_f$ changes on flash illumination when electron flow through them can occur. PS II may be inhibited easily with DCMU and or/dithionite. The P-518$_f$ change observed in the presence of DCMU, dithionite and an uncoupler is due to electron flow through PS I and thus provides a measure of the PS I contribution to the P-518$_f$ change.

The P-518$_s$ change is due to electron flow through the cytochrome *b-f* complex when certain redox conditions are met; the driving force for this reaction is provided by PS I via plastocyanin and cytochrome *f* and the maximal amplitude of P-518$_s$ is thus equal to the amplitude of the P-518$_f$ change due to PS I electron transfer. The extent of P-518$_s$ is influenced by the availability of oxidant (cytochrome f^+) and reductant (PQH_2) at the cytochrome *b-f* complex and Δp (in chloroplasts as ΔpH or luminal pH). With due care, the extents of all these changes may be used to obtain estimates of the relative rates of linear and cyclic electron flows.

Examples

For Fig. 1 the PS I contribution to P-518$_f$ is the extent of P-518$_f$ in the rightmost trace (' + dithionite'), equal to $\Delta I/I$ of $4 \cdot 10^{-3}$. In the third trace from the left (' + 2 μM nigericin'), the extent of P-518$_s$ is also $4 \cdot 10^{-3}$ ($\Delta I/I$), indicating maximal operation of the 'extra' $\Delta\psi$ generator at the cytochrome *b-f* complex. The PS I contribution to $\Delta I/I$ P-518$_f$ must also be $4 \cdot 10^{-3}$, leaving a $\Delta I/I$ P-518$_f$ due to PS II of $1.53 \cdot 10^{-3}$. The ratio of PS II activity to PS I activity is thus 1.53 : 4 or 0.38 : 1. As these measurements were made under 'pseudo steady-state' conditions where each flash elicits a similar response, the predominant electron-flow pattern in this case is clearly PS I-driven cyclic electron flow. The ratio of PS I cyclic to linear

electron flow is $(1 - 0.38) : 0.38$ or $1.63 : 1$.

In the fourth trace from the left ('+2 mM oxaloacetate'), the PS I $\Delta I/I$ P-518$_f$ contribution is again $4 \cdot 10^{-3}$, but the extra P-518$_f$ due to PS II activity is equal to $\Delta I/I$ of $2.83 \cdot 10^{-3}$, giving a ratio of cyclic to linear electron flows of $(4 - 2.83) : 2.83$ or $0.41 : 1$. The extent of P-518$_s$ is diminished here because the electron-transfer chain was in a more oxidised state due to the presence of an electron acceptor (as the plastoquinone pool is oxidised the extent of P-518$_s$ diminishes). The ratio of P-518$_s$:PS I P-518$_f$ here is about $0.33 : 1$, compared with a ratio of $1 : 1$ in the '+nigericin' trace.

For Fig. 2 no absolute measure of the PS I contribution to P-518$_f$ is available, but examination of Fig. 2 (upper centre trace) shows that, for the aerobic sample, it must be greater than or equal to $1.54 \cdot 10^{-3}$ $\Delta I/I$ (the extent of P-518$_s$). Subtraction of this contribution leaves a PS II-derived P-518$_f$ $\Delta I/I$ of less than or equal to $0.86 \cdot 10^{-3}$, giving PS II activity:PS I activity less than or equal to $0.56 : 1$ and a ratio of cyclic to linear electron flows of greater than or equal to $(1.54 - 0.86) : 0.86$ or $0.79 : 1$. On addition of oxaloacetate (upper right trace), P-518$_f$ increased to give a ratio of PS II activity to PS I activity of less than or equal to $1 : 1$. The P-518$_s$ here was much smaller than in the previous trace. These data show that switching between linear and cyclic electron flows undoubtedly occurs. While P-518$_s$ generation by a Q-cycle in linear electron flow might occur, it would require the maintenance of an extremely tight balance of electron input and output to stay within the narrow range of redox potentials necessary for its operation. Such seems unlikely from the data presented here and from theoretical considerations [47].

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ENERGETICS OF PHOTOSYNTHESIS IN *ZEA MAYS*

II. STUDIES OF THE FLASH-INDUCED ELECTROCHROMIC SHIFT AND FLUORESCENCE INDUCTION IN MESOPHYLL CHLOROPLASTS

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Patterns of electron transfer in isolated mesophyll chloroplasts of maize (*Zea mays* L.) were studied in the presence of the physiological substrates, oxaloacetate, 3-phosphoglycerate and pyruvate. Flash-induced absorbance changes due to the electrochromic pigment band-shift (P-518) were used to estimate relative electron flow rates through the cyclic and non-cyclic pathways of electron transport. Further information on the redox state of electron carriers and the activity of coupled electron flow was obtained from measurements of fluorescence induction and of actinic-light-induced fluorescence changes. The results demonstrate the importance of correct redox poising for optimal rates of photosynthesis and are discussed in relation to the operation and regulation of photosynthesis in the C_4 system.

Introduction

The pathway of photosynthetic carbon assimilation in C_4 plants such as maize has been elucidated through studies both of enzyme localisation [1–4] and of the photosynthetic capacities of isolated mesophyll protoplasts and chloroplasts and bundle sheath strands [5–8]. The cytoplasm of the mesophyll cells is the site of carboxylation of phospho*enol*pyruvate and the resulting oxaloacetate is reduced to malate in the mesophyll chloroplasts. Malate is then transferred to the bundle sheath where it is decarboxylated to yield CO_2, NADPH and pyruvate. The pyruvate returns to the mesophyll chloroplast and is phosphorylated to regenerate phospho*enol*pyruvate, completing the cycle. Since the bundle sheath chloroplast is deficient in PS II activity [4,7–11] and malate decarboxylation generates only half of the NADPH requirement for 3-phosphoglycerate reduction, up to half of the 3-phosphoglycerate formed in the Benson-Calvin cycle must be exported from the bundle sheath to the mesophyll chloroplast for conversion to triose phosphate and then returned to the bundle sheath, forming a subsidiary cycle.

When the energetic requirements of this pathway are considered, it is evident that the fixation of one molecule of CO_2 into triose phosphate

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Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Mes, 2-(*N*-morpholino)ethanesulphonic acid; Tricine, *N*-tris(hydroxymethyl)methylglycine; F_0, intrinsic bed fluorescence; F_i, initial level of fluorescence seen during rapid induction (F_0 plus a variable component proportional to the state of reduction of Q); F_m, maximal level of fluorescence (seen in the presence of DCMU); F_v, variable fluorescence (maximally $F_m - F_0$); Chl, chlorophyll; PS, photosystem.