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ENERGETICS OF PHOTOSYNTHESIS IN *ZEA MAYS*

I. STUDIES OF THE FLASH-INDUCED ELECTROCHROMIC SHIFT AND FLUORESCENCE INDUCTION IN BUNDLE SHEATH CELLS

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Bundle sheath strands free of mesophyll contamination were isolated from 3–4-week-old leaves of maize (*Zea mays* L.). Patterns of electron flow in the preparations were studied in the presence of physiological substrates. Relative electron flow rates were estimated from the flash-induced electrochromic band shift changes (P-518) and cytochrome *f* turnover. Induction of chlorophyll fluorescence was also measured. Little Photosystem II activity was found to be present, the principal pathway of electron flow being Photosystem I-driven cyclic electron transfer. The latter was activated through reductive poising by NADPH, generated via malate decarboxylation or (less efficiently) from dihydroxyacetone phosphate. The actions of these electron donors and of oxygen, nitrite and methyl viologen as electron acceptors in redox poising the Photosystem I-driven cycle were investigated and are discussed in relation to the regulation of photosynthesis in the bundle sheath.

Introduction

In mature leaves of maize, C_4 photosynthesis involves the condensation of CO_2 with phospho*enol*pyruvate, in the mesophyll cells, to yield oxaloacetate; this is reduced to malate which is transported to the bundle sheath and there is decarboxylated by NADP-malic enzyme to yield CO_2, NADPH and pyruvate. Pyruvate then returns to the mesophyll, where it is reconverted to phospho*enol*pyruvate [1].

In the bundle sheath, CO_2 is fixed by reaction with ribulose 1,5-bisphosphate to yield 3-phosphoglycerate (as in C_3 photosynthesis) and the 3-phosphoglycerate is reduced to triose phosphate at the expense of NADPH. Since malate decarboxylation provides sufficient NADPH to reduce no more than half of the 3-phosphoglycerate formed, reduction of the remaining 3-phosphoglycerate must be driven by reductant from an alternative source. Bundle sheath chloroplasts are deficient in

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Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; Tricine, *N*-tris(hydroxymethyl)methylglycine; PVP, polyvinylpyrrolidone; DBMIB, 2,5-dibromo-3-methyl-6-isopropylbenzoquinone; DNP-INT, 2,4-dinitrophenyl ether of iodonitrothymol; 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, maximum level of fluorescence (seen in the presence of DCMU); F_v, variable fluorescence (maximally $F_m - F_0$); Chl, chlorophyll; PS, photosystem.

PS II; estimates of the degree of this deficiency made using artificial electron acceptors differ considerably [2–10], but in experiments where the natural acceptor, CO_2, is employed there can be little doubt that NADPH formation dependent upon linear electron flow is very limited [11,12]. Thus, 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 reduction. Triose phosphate then returns to the bundle sheath chloroplast in order to maintain sugar phosphate pools.

In the extreme case where the bundle sheath completely lacks PS II, the fixation of one molecule of CO_2 into triose phosphate will require the generation of two molecules of ATP in the bundle sheath chloroplast, this requirement being largely met by cyclic photophosphorylation [12,13]. Cyclic electron flow is very sensitive to redox poise and requires activation by a suitable reductant; this poising is accomplished by PS II [14,15] and O_2 [16,17] or by added reductant [18]. Such PS II activity as exists in the bundle sheath is demonstrably insufficient to poise cyclic electron flow and instead NADPH, generated by malate decarboxylation, provides the necessary reducing power [13].

NADPH may influence such residual PS II activity as may be present in bundle sheath strands; it has been shown that incubation of spinach thylakoids with NADPH in the dark results in partial reduction of the primary acceptor PS II and of the plastoquinone pool [19,20] and it has been suggested that closure of PS II traps by this mechanism could decrease linear electron flow and thereby stimulate cyclic photophosphorylation [19]. In the bundle sheath, such a mechanism could clearly operate to reduce further an already low input of electrons from PS II.

This paper provides further evidence of the low activity of PS II in mature bundle sheath cells showing high rates of CO_2 fixation and suggests mechanisms whereby the bundle sheath chloroplast may optimise its ATP production when its natural substrates are present.

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. Percoll was obtained from Pharmacia, Uppsala, Sweden. Nigericin was kindly given by Dr. R.L. Hamill of Eli Lilly Laboratories.

Preparation of bundle sheath strands. Strands of bundle sheath cells were prepared by a modification of the procedure of Chapman et al. [12]. Deribbed leaves from 3–4-week-old plants were sliced into 0.5–1.0 mm segments and blended in a Waring blender (150 ml capacity, 30 s at 50 V (110 V line voltage) in a medium containing 0.35 M sorbitol, 4 mM $MgCl_2$, 2 mM KH_2PO_4, 10 mM sodium isoascorbate, 20 mM Hepes-KOH (pH 6.4). The remaining tissue was collected on Miracloth, washed with 150 ml of the same medium and suspended in 50 ml of the same medium containing 2% (w/v) cellulase (Onozuka 3S), 0.3% (w/v) pectinase (Macerase) and 1% (w/v) PVP-40. The tissue was incubated at 28°C under illumination for 25 min, collected by filtering on Miracloth, washed with 50 ml of the above medium (adjusted to pH 8.0) and blended for 15 s at 60 V. The strands were washed and resuspended in 0.35 M sorbitol, 4 mM $MgCl_2$, 2 mM KH_2PO_4, 5 mM K_2SO_4, 10 mM Tricine-KOH (pH 8.2) and then centrifuged at $100 \times g$ for 30 s. The pellet was resuspended in the same medium at three-quarter strength. For flash studies the medium also contained 30% (v/v) Percoll and 2% (w/v) PVP-360 (mixed just prior to use) while for measurements of chlorophyll fluorescence it contained 40% (v/v) Percoll. Microscopic examination of isolated strands showed them to be completely free of mesophyll contamination. This method yielded preparations of bundle sheath strands capable of physiological rates of CO_2 fixation [12,13].

Spectrophotometry. Flash-induced absorbance changes at 518 and 554 nm were recorded and analysed in a single-beam specrophotometer linked to a PDP11/34A computer as described previously [21]. The actinic xenon flash (duration 4 μs at half amplitude) was filtered through a Schott RG 665 filter. The flash frequency was 2 Hz. Traces represent the average of 256 records.

Chlorophyll fluorescence. Chl *a* fluorescence was excited by a weak (2.2 $W \cdot m^{-2}$) measuring beam obtained by filtering the output of a mercury lamp

through Corning 4-96 and Balzers DT-blau filters. Fluorescence induction curves were recorded using a Fabritek 1052 digitiser. Shutter opening time on the instrument was 3 ms (10–90%). Fluorescence at 685 nm was detected after transmission through a Bausch and Lomb 0.5 m monochromator blocked with a Corning 2-58 cut-off filter.

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 [22].

Results

P-518 measurement

P-518 changes during a series of repetitive actinic flashes were used to provide a convenient monitor of electron flow. The flash power, duration and frequency used here correspond to a low average intensity of illumination and the results may be used to indicate the patterns of electron flow which will occur under such intensities of steady-state illumination. The fast (nanosecond) changes ($P\text{-}518_f$) arise from electron flow through the PS I and PS II reaction centres [23], while slow (millisecond) changes ($P\text{-}518_s$) indicate a further charge separation during intersystem electron flow that is seen only at suitably low redox poise [21,24]. During the 'pseudo steady-state' illumination provided by repetitive flashes the relative extents of $P\text{-}518_f$ and $P\text{-}518_s$ may be used to estimate the activities of non-cyclic and of PS I-driven cyclic electron flows; non-cyclic electron flow to an acceptor will lead to a large $P\text{-}518_f$ (full electron transfer through both reaction centres) and a small $P\text{-}518_s$ (as intersystem pools are largely oxidised), while increasing turnover of PS I-driven cyclic electron flow will be accompanied by a decrease in $P\text{-}518_f$ (as PS II acceptors become reduced) and a large $P\text{-}518_s$. When PS II is absent or inoperative, the extent of $P\text{-}518_s$ equals the extent of (PS I-derived) $P\text{-}518_f$ [21].

P-518 in bundle sheath strands

Previous work showed that cyclic electron flow is only induced by adding malate to bundle sheath strands and is then insensitive to DCMU, whereas little electron flow is observed in the absence of malate, leading to the conclusion that electrons provided by PS II are neither effective nor are they required for redox poising of the PS I cycle in this tissue [13]. The results shown in Fig. 1 (upper traces) demonstrate poising of cyclic electron flow by malate following DCMU addition. The addition of CO_2 and then of nigericin to bundle sheath strands was accompanied by some reaction centre turnover, as indicated by $P\text{-}518_f$; with this sample under aerobic conditions P-518 was somewhat suppressed by DCMU, but was restored by subsequent addition of malate, when it was accompanied by a substantial $P\text{-}518_s$. $P\text{-}518_s$ was then fur-

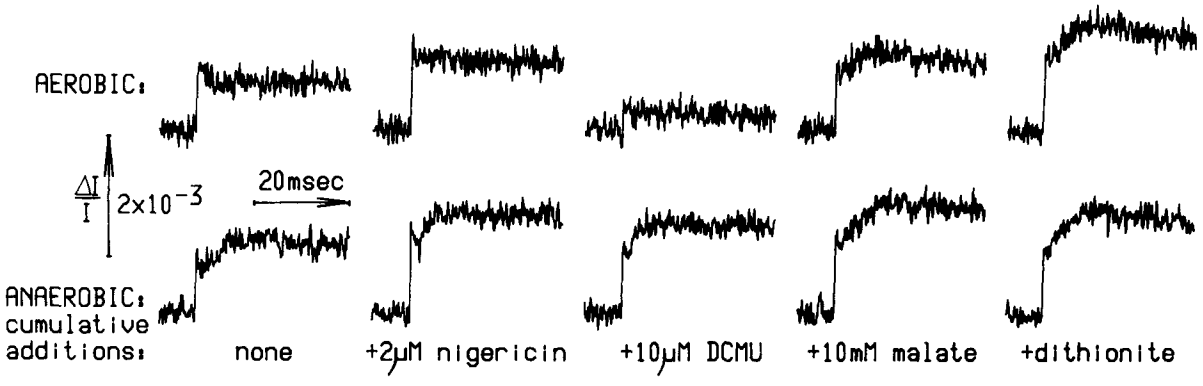

Fig. 1. Flash-induced absorbance change at 518 nm in bundle sheath strands. The reaction mixture (1.5 ml) contained 0.26 M sorbitol, 3 mM $MgCl_2$, 1.5 mM KH_2PO_4, 3.75 mM K_2SO_4, 7.5 mM Tricine-KOH (pH 8.2), 30% (v/v) Percoll, 2% (w/v) PVP-360, 10 mM $NaHCO_3$ and bundle sheath strands equivalent to 57 μg Chl. The lower series of samples was made anaerobic as described in Materials and Methods. Sequential additions were made as shown.

ther enhanced by the addition of dithionite. The extent of P-518$_f$ after DCMU/dithionite addition was greater than 92% of the P-518$_f$ extent before DCMU addition. As the pre-DCMU trace includes both PS II- and PS I-derived changes while the post-DCMU traces reflect PS I-derived changes only, the PS II/PS I ratio must be less than 8 : 92. This small amount may still be enough to overcome loss of electrons to oxygen from PS I acceptors, accounting for the effect of DCMU observed here.

Cytochrome *f* turnover was also measured under similar conditions (Fig. 2, upper traces). There was no detectable cytochrome *f* turnover in the presence of CO_2 and nigericin despite the appreciable P-518$_f$, which indicates that electron donation from PS II was insufficient to reduce cytochrome *f* between flashes and provides further evidence of the very limited contribution of electrons by PS II in this tissue. Subsequent addition of malate (following DCMU) resulted in cytochrome *f* turnover (i.e., reduction of cytochrome *f* between flashes) which was enhanced by the addition of dithionite.

Since redox poising of cyclic electron flow in the bundle sheath is considered to depend upon electrons donated by malate via NADPH [13], the influence of malate might be expected to be sensitive to the O_2 concentration, as O_2 readily oxidises ferredoxin in chloroplasts [25–27]. Figs. 1 and 2 (lower traces) show the effect of anaerobiosis on both P-518 and cytochrome turnover with the same additions as under aerobic conditions. The most striking feature was that under anaerobiosis cyclic electron flow (indicated by the presence of P-518$_s$ and cytochrome *f* turnover) proceeded readily without any additions to the bundle sheath strands other than CO_2. When nigericin was added there was a large increase in signal amplitude together with an enhancement of P-518$_s$ which suggests that, under anaerobiosis, the bundle sheath cells contained sufficient endogenous reductant (malate and/or NADPH) and that reductant which was generated was not diverted to reduce O_2. The efficiency of donation by endogenous reductant was exemplified by the marginal sensitivity to DCMU in the absence of O_2. Subsequent addition of malate led to a further increase in amplitude and a more pronounced P-518$_s$ (considerably greater than that observed under aerobic conditions) and there was no further enhancement by dithionite, showing that an optimal state of reduction had already been achieved.

Fig. 3 shows the sensitivity of P-518 in bundle sheath strands to the electron acceptor methyl viologen in anaerobic as well as aerobic suspensions. The bundle sheath cell is permeable to methyl viologen [10] which is an effective electron sink under aerobic conditions because it catalytically transfers electrons to O_2. Fig. 3a shows that under aerobic conditions, addition of malate and nigericin resulted in the appearance of the slow phase of P-518, accompanied by a large fast phase. However, the addition of methyl viologen resulted not only in removal of P-518$_s$ but also in a very large decrease in the amplitude of P-518$_f$. The

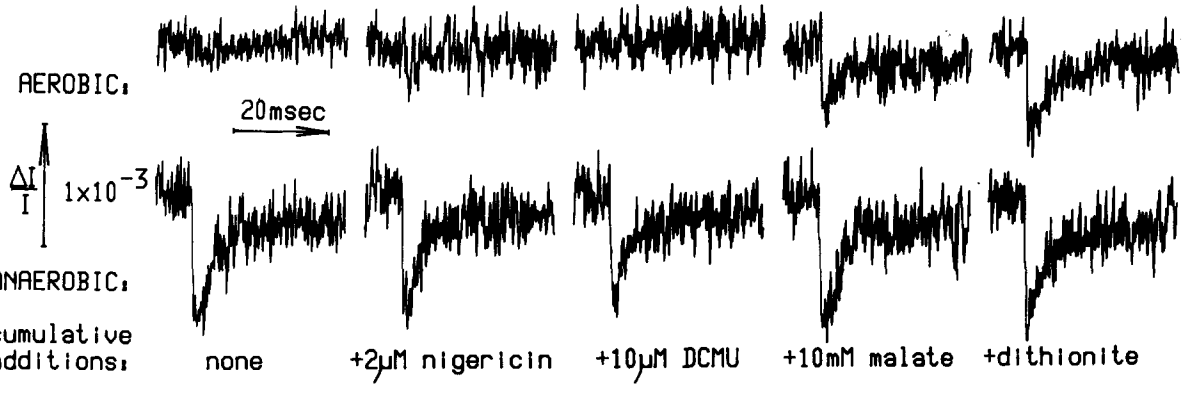

Fig. 2. Flash-induced cytochrome *f* turnover in bundle sheath strands. Reaction conditions were as described in the legend to Fig. 1, except that the chlorophyll concentration was 44 μg Chl in 1.5 ml. Sequential additions were made as shown.

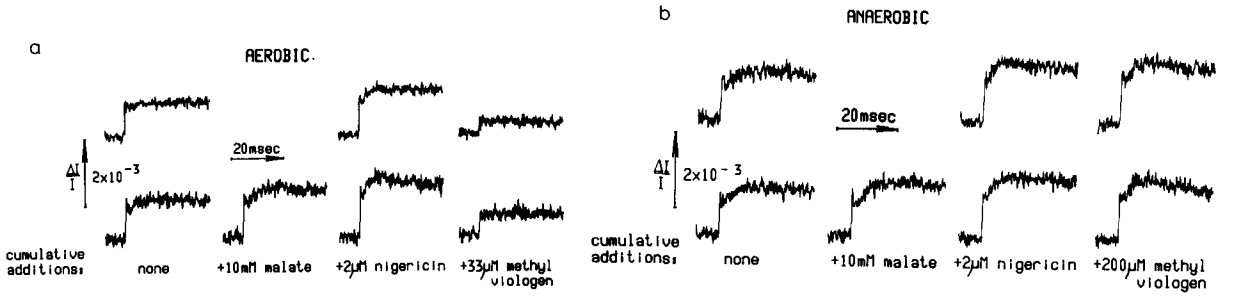

Fig. 3. Flash-induced absorbance change at 518 nm in (a) aerobic and (b) anaerobic suspensions of bundle sheath strands. Reaction conditions were as described in the legend to Fig. 1, except that the chlorophyll concentration was 58 μg Chl in 1.5 ml. Sequential additions were made as shown; malate was included in the lower series of traces and omitted in the upper series. Reaction mixtures were made anaerobic as described in Materials and Methods.

latter was in contrast to the effect of methyl viologen in spinach chloroplasts in which it eliminates P-518$_s$ by competing with cyclic electron flow for electrons and, in the presence of an uncoupler, increases the amplitude of P-518$_f$ due to the stimulation of linear electron flow (a similar effect may be seen when an efficient electron acceptor such as oxaloacetate is added to maize mesophyll chloroplasts [28]). Fig. 3b shows the contrasting results obtained under anaerobic conditions. Addition of malate and nigericin led to the expected P-518 signal but subsequent addition of methyl viologen, even at a concentration of 200 μM, had no detectable effect, showing that when oxygen was absent any electron withdrawal from the cycle by methyl viologen was matched by electron donation from malate, i.e, when methyl viologen cannot donate to oxygen, it does not drain electrons from the cyclic pathway to any great extent.

Triose phosphate is potentially an alternative source of NADPH in chloroplasts. It has recently been shown that in wheat chloroplasts dihydroxyacetone phosphate can reduce NADP, ferredoxin and thioredoxin through a reversal of the reaction catalysed by glyceraldehyde-phosphate dehydrogenase [26]. This electron donation by dihydroxyacetone phosphate was very sensitive to O_2, presumably because reduced ferredoxin is directly oxidised by O_2 [29]. Dihydroxyacetone phosphate (which is also a key metabolite in the bundle sheath) was added to bundle sheath strands to ascertain whether it could act as a source of reductant. Fig. 4 shows that, under aerobic conditions, the addition of dihydroxyacetone phosphate caused only a small increase in P-518$_s$. Addition of nigericin increased the extent of both phases of P-518 as expected. Addition of DCMU removed P-518$_s$ and substantially decreased P-518$_f$, indicating that electron donation by dihydroxyacetone phosphate was insufficient to maintain an adequately low redox poise under these conditions. Under anaerobic conditions, in the presence of dihydroxyacetone phosphate, P-518$_s$ was increased over that seen with CO_2 alone and addition of nigericin led to a further increase in signal size (again compare Figs. 1 and 3). This signal then showed only slight sensitivity to DCMU, suggesting that the decrease seen under aerobic conditions was due to a bleeding of electrons to O_2.

Fig. 5 illustrates two further characteristics of P-518$_s$ in the bundle sheath. In Fig. 5a the effect of adding a physiological electron acceptor to the bundle sheath strands is shown. The maximum P-518$_s$ was elicited by adding CO_2, malate and nigericin under anaerobic conditions. Subsequent addition of nitrite resulted in removal of P-518$_s$, although unlike the addition of methyl viologen to an aerobic bundle sheath suspension (Fig. 3), it did not diminish P-518$_f$, indicating that in these circumstances it was a relatively inefficient electron acceptor. This observation, especially considering the high concentration of nitrite required, may reflect poor permeability of the bundle sheath cells to nitrite and/or a low activity of nitrite reductase in the bundle sheath [30]. Oxaloacetate was found to have no effect on P-518 in the bundle sheath (data not shown), consistent with

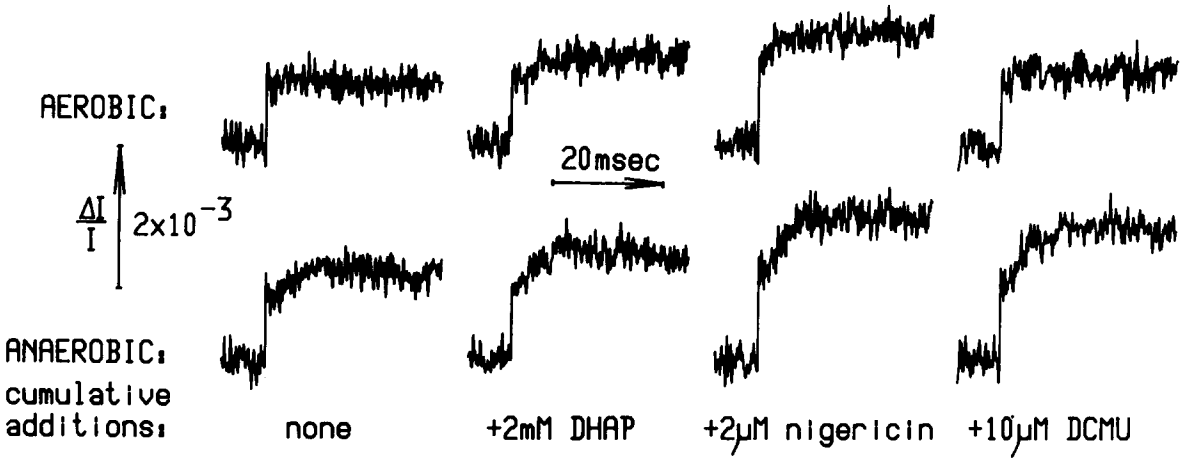

Fig. 4. Flash-induced absorbance change at 518 nm in bundle sheath strands. Reaction conditions were as described in the legend to Fig. 1, except that the chlorophyll concentration was 71 μg Chl in 1.5 ml. The lower series of samples was made anaerobic as described in Materials and Methods. Sequential additions were made as shown. DHAP, dihydroxyacetone phosphate.

the very low activity of malate dehydrogenase found in these cells [31,32].

In Fig. 5b, the influence of the quinone analogue, DBMIB, is shown. As in spinach chloroplasts DBMIB removed P-518$_s$ [21], indicating the involvement of plastoquinone in cyclic electron flow in the bundle sheath. Similar effects were observed by adding other quinone analogues such as DNP-INT [33] and 2-pivaloyl-1,3-indanedione.

Previous investigations of bundle sheath photosynthesis have shown that both AMP and aspartate stimulate CO_2 fixation [12,34]. In these studies we found that AMP had no effect on P-518 and did not stimulate CO_2 fixation. Aspartate, on the other hand, sometimes promoted a slight enhancement of P-518$_s$, which might indicate enhanced generation of reductant (for instance, by aspartate acting as a source of reductant in the bundle sheath chloroplast at the expense of cytosolic NADH [34]).

P-518$_s$ could be observed in the presence of malate and nigericin in isolated intact bundle sheath chloroplasts (results not shown), although such chloroplasts only exhibit rates of CO_2 fixation of about 20 μmol/h per mg Chl at 20°C (unpublished results) and were therefore unsuitable for more extensive studies.

Chlorophyll fluorescence in bundle sheath strands

In C_3 chloroplasts fast changes (occurring within a few seconds) in the induction of chlorophyll fluorescence are characterised by a sigmoidal rise from a level, F_i, to a variable level during illumination and reflect a transition from a state where Q

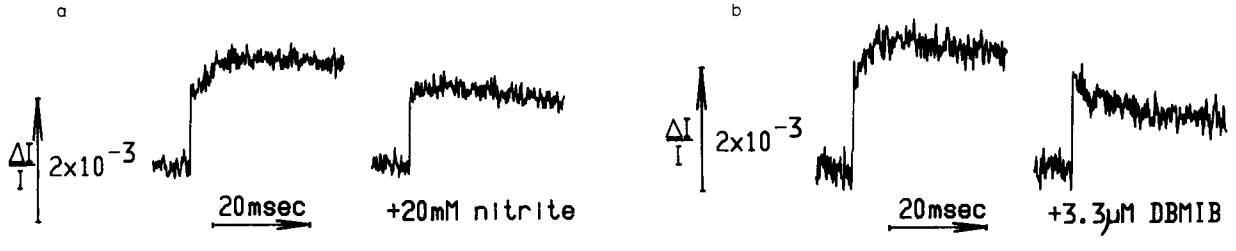

Fig. 5. Influence of nitrite (a) and DBMIB (b) on the flash-induced absorbance change at 518 nm in bundle sheath strands. Reaction conditions were as described in the legend to Fig. 1 except that the reaction medium was supplemented with 10 mM malate and 2 μM nigericin. The chlorophyll concentration was 43 μg Chl in 1.5 ml. Nitrite or DBMIB was added following the first measurement.

is oxidised to one in which it is transitorily reduced [35]. The magnitude of F_i depends upon the intrinsic bed fluorescence, F_0, and upon the extent of Q reduction in the dark (the latter governed by the reduction state of the plastoquinone pool, intersystem pools of electron acceptors and the ratio NADPH/NADP). The area above the fast fluorescence induction curve is a measure of the size of the pool of oxidised electron carriers which can reoxidise Q [36].

Measurement of fluorescence induction in bundle sheath strands over a period of 20 s showed three principal features. First, the overall fluorescence yield was about 10-times lower than that from maize mesophyll or spinach chloroplasts at a comparable chlorophyll concentration. Second, in separate experiments, little fluorescence increase was seen when actinic illumination (25 $W \cdot m^{-2}$) was superimposed on a weaker (0.1 $W \cdot m^{-2}$) modulated probe beam, unlike results obtained with maize mesophyll [28] or spinach chloroplasts. Third, maximum fluorescence observed in the presence of DCMU was only about twice the level of F_i, whereas in mesophyll chloroplasts [28] and spinach chloroplasts [37] the ratio F_v/F_i is about 5. Previous reports of fluorescence induction in bundle sheath chloroplasts [37] and thylakoids [7,9] show a similar ratio of F_v to F_i, although Gregory et al. [38] claimed that bundle sheath cells fail to show any fluorescence induction.

Fig. 6a shows that without any additions there was a relatively slow increase from F_i to a low final level of fluorescence (the levels of F_i marked on the traces were determined from the rapid fluorescence rise, measured about 3.8 ms after the start of shutter opening in separate fluorescence induction curves measured with a 200 ms time base). This 20 s increase in fluorescence showed very little sigmoidal character (see also Refs. 35–37). Preincubation with malate in the dark under aerobic conditions resulted in both a modest increase in F_i and a tendency for the induction curve to become more hyperbolic, with a rapid rise to the final fluorescence intensity which was sometimes a little higher (results not shown). If the bundle sheath strands were preincubated in the dark under anaerobic conditions with malate and dihydroxyacetone phosphate (i.e., conditions which would be expected to lead to a more efficient

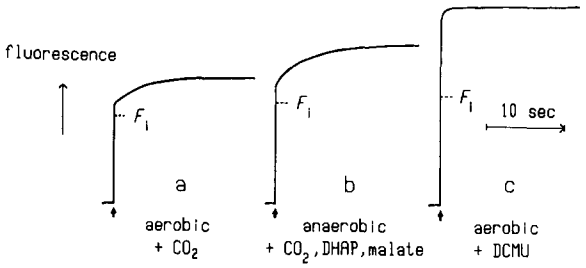

Fig. 6. Induction of chlorophyll fluorescence in a suspension of bundle sheath strands. Fluorescence was excited and measured as described in Materials and Methods. The reaction mixture (3 ml) contained 0.26 M sorbitol, 3 mM $MgCl_2$, 1.5 mM KH_2PO_4, 7.5 mM Tricine-KOH (pH 8.2), 40% (v/v) Percoll and bundle sheath-strands equivalent to 57 μg Chl. The traces were obtained from three separate samples of dark-adapted bundle sheath strands. Concentrations of the additions were: CO_2, 10 mM $NaHCO_3$; dihydroxyacetone phosphate (DHAP), 2 mM; malate, 10 mM; DCMU, 10 μM. Sample b was made anaerobic as described in Materials and Methods.

generation of reductant) (Fig. 6b), there was a further increase in F_i, a more rapid fluorescence rise and a higher maximal fluorescence intensity, the latter still, however, below that obtained in the presence of DCMU (Fig. 6c). Conversely, addition of strong oxidants (with or without pre-illumination) such as nitrite and methyl viologen gave no perceptible change from the fluorescence induction curve measured in the presence of CO_2 alone (results not shown), suggesting that the F_i level with CO_2 alone was very close to F_0.

These results are compatible with only a low amount of PS II in the bundle sheath and concur with previous measurements of 77 K fluorescence emission spectra which show very low levels of PS II fluorescence [39].

Discussion

The question of the PS II content of the bundle sheath in plants such as maize has been the subject of good deal of controversy. The results presented in this paper provide further evidence for the conclusion that PS II is essentially inactive in maize bundle sheath and that malate sythesised in the mesophyll tissue is primarily responsible, through NADPH, for redox poising and initiation of cyclic electron flow. Although Hatch and Osmond [1] have pointed out that there can be little doubt that photochemical generation of

NADPH is considerably less than half of that required to support measured rates of light-saturated photosynthetic carbon reduction, claims of high oxygen-evolving capacity in maize bundle sheath preparations have been made subsequently [9,10]. Golbeck et al. [37] have recently reassessed the content of PS II in bundle sheath chloroplasts of maize and concluded that they have only 15–20% of the oxygen-evolving capacity of mesophyll chloroplasts (i.e, a rate of 150–200 μequiv./h per mg Chl in the bundle sheath chloroplasts); they placed particular emphasis on the need for bundle sheath preparations which are free of mesophyll contamination. However, the important question relates not to the maximal PS II content of the bundle sheath measured in the presence of artificial electron donors and acceptors but to how much of the potential PS II activity is expressed in chloroplasts or cells which fix CO_2 at physiological rates. Recent work by Chapman et al. [12] shows that the rate of O_2 evolution by maize bundle sheath strands fixing CO_2 at high rates (200 μmol CO_2/h per mg Chl) is less than 20 μmol O_2/h per mg Chl and that linear electron flow could provide, at most, about 10% of the NADPH required for reduction of the 3-phosphoglycerate formed during photosynthesis.

The following observations, presented in this and a previous paper [13], support the conclusion that PS II is essentially inactive in the functional bundle sheath:

(i) The insensitivity of cytochrome turnover and the electrochromic effect to DCMU in the presence of a suitable reductant such as malate (Ref. 13 and Figs. 1, 2 and 4) indicates that PS II is not a significant donor of the reducing power required for activation of efficient cyclic electron flow.

(ii) The sensitivity of electron flow in aerobic bundle sheath strands to methyl viologen (Fig. 3a) and the minimal turnover seen in the absence of an added reductant (Ref. 13 and Figs. 1–4) suggest that in the absence of malate or other suitable donors the electron-transport system is readily over-oxidised and therefore that the PS II contribution must be very small. A similar effect of an oxidant (oxaloacetate) was seen in maize mesophyll chloroplasts only when PS II was blocked with DCMU [28].

As might be expected, the differing sensitivity to DCMU in the presence and absence of malate is observed for CO_2 fixation as well as for P-518. Farineau [40,41] and Chapman et al. [12] have shown that CO_2 fixation in bundle sheath strands is markedly less sensitive to DCMU in the presence of malate. A similar conclusion has been reached by Rathnam and Edwards [42] using bundle sheath chloroplasts of another NADP-malic enzyme species, *Digitaria sanguinalis*.

Some measurements of P-518 in maize bundle sheath chloroplasts have been made in previous investigations. Roux and Faludi-Daniel [43] and Horvath et al. [44] concluded that $P\text{-}518_f$ was only observed in bundle sheath chloroplasts upon the addition of phenazine methosulphate (an artificial catalyst of electron flow around PS I which would not lead to the generation of $P\text{-}518_s$) or of 'mesophyll extract'. Hence they concluded that 'silent' PS I was activated in the bundle sheath. However, in none of these experiments were physiological substrates supplied, nor did the authors provide adequate evidence of the photosynthetic competence of their preparations.

As in spinach chloroplasts and leaves [45–47], cyclic electron flow in the bundle sheath is sensitive to the presence of O_2, which presumably competes, at the level of ferredoxin, with cyclic electron flow for electrons donated by NADPH. However, $P\text{-}518_s$ is enhanced under anaerobic conditions and there is no evidence that over-reduction occurs (CO_2 fixation in bundle sheath strands is inhibited little by anaerobiosis [12]) in contrast to the PS II-containing spinach chloroplast [47]. Photosynthesis in the bundle sheath in vivo may thus be enhanced by a lowering of the O_2 tension and inhibited by a high O_2 tension, particularly if reductant is limiting. Such a possibility prompts speculation regarding the concentration of O_2 in the bundle sheath, particularly as a substantial oxidation of NADPH by O_2 would disrupt the stoichiometry of the C_4 cycle. Raven [48] considers that in the bundle sheath of NADP-malic enzyme species, O_2 evolution is likely to be equalled by respiratory and photorespiratory O_2 uptake and that the bundle sheath will be approximately in equilibrium with the air concentration of O_2. Measurements of O_2 exchange in isolated bundle sheath strands also suggest that O_2 uptake does not greatly exceed O_2 evolution, net O_2 uptake being about

5% of the rate of photosynthesis [12]. However, even a modest uptake of O_2 in vivo could be significant if the bundle sheath cell wall presents a substantial barrier to the diffusion of O_2. Thus, of the estimates of the magnitude of the diffusion resistance of the bundle sheath cylinder [1,48,49], the highest would allow an appreciable concentration gradient of O_2 to develop between the mesophyll and bundle sheath as a result of even slight net O_2 uptake. The possibility that the bundle sheath is partially anaerobic cannot be discounted.

The observation that P-518$_s$ is enhanced by dihydroxyacetone phosphate (Fig. 4) lends further support to the conclusion that NADPH is the poising agent in the bundle sheath chloroplasts. Such a conclusion raises many questions in regard to regulation of photosynthesis in the bundle sheath. One aspect is the extent to which NADP-malic enzyme activity may be enhanced in vivo by increased stroma pH and Mg^{2+} in the light [50,51] because if the enzyme is tightly regulated by such mechanisms its activity could, in turn, be dependent upon cyclic electron flow to drive the H^+ and Mg^{2+} fluxes responsible. Measurements of CO_2 release and pyruvate production in isolated bundle-sheath strands of maize and *D. sanguinalis* provide conflicting evidence, suggesting that malate decarboxylation is both light independent [52,53] and fully light dependent [11,12]. If the latter claim is correct, then either regulation of NADP-malic enzyme is not so rigorous as to preclude the generation of a favourable NADPH/NADP ratio in the dark and thereby to activate electron transport immediately upon illumination (a view which is supported by the fluorescence induction measurements of Fig. 6), or else the chloroplast must contain sufficient residual endogenous NADPH in vivo [54]. It has been proposed that the enzymes of the Benson-Calvin cycle which exhibit reductive activation are reduced by electrons from NADPH [26] and that activation of these enzymes would therefore depend upon prior decarboxylation of malate.

Poising of cyclic electron flow by NADPH might also be seen to be in conflict with the view and observation that 3-phosphoglycerate is produced in excess of the capacity of the bundle sheath chloroplast to reduce it [8,11,12]. However, the amount of glycerate 1,3-bisphosphate available for reduction would be regulated by the ATP/ADP ratio [55]. Thus, as the NADPH/NADP ratio decreased, the electron carriers mediating cyclic electron flow would shift away from optimal poise and hence the ATP/ADP ratio would fall, resulting in a decreased conversion of 3-phosphoglycerate (the ATP/ADP ratio would also affect 3-phosphoglycerate formation by its effect on the ribulose-5-phosphate kinase reaction [56]). Such a system would thus attain a balance, limiting NADPH consumption and allowing ATP production. The ATP/ADP ratio might therefore play a role in regulation of the relative contributions of the bundle sheath and mesophyll to reduction of the 3-phosphoglycerate formed.

From the characteristics of P-518$_s$ in bundle sheath strands it is possible to deduce the relative efficiency of donation of electrons to the cyclic pathway under particular conditions. Thus, most efficient donation occurs under anaerobic conditions and in the presence of exogenous reductants, malate and/or dihydroxyacetone phosphate. These conditions would hence be expected to lead to a high NADPH/NADP ratio and thereby effect a reduction of the plastoquinone pool and the PS II electron acceptors. However, the increases in fluorescence under these conditions are modest when compared with the increases seen on incubation of spinach thylakoids with NADPH [19] (in which NADP has been shown to be very inhibitory to the NADPH-induced fluorescence increase). The above observations in the bundle sheath may be attributable to two processes. First, NADPH generation by NADP-malic enzyme may be impaired in the darkened chloroplast, as discussed above. However, pre-illumination of bundle sheath strands in actinic light, although leading to an increase in the rate of transition from F_i to F_v in a subsequent fluorescence induction, did not substantially increase F_i or F_v, suggesting that this is not a fully adequate explanation (nor was there a detectable increase in fluorescence during actinic illumination). Second, the low fluorescence yield in the presence of a reductant relative to the fluorescence yield in the presence of DCMU (compare, for example, mesophyll chloroplasts in the presence of DCMU and of pyruvate [28]) may reflect a sluggisahness in the reduction of the PS II electron

acceptors by electrons from water-splitting, indicating that the rate of oxidation of PS II acceptors by intersystem pools and plastoquinone far exceeds the capacity for their reduction by PS II. Nevertheless, malate, despite its apparently minor effect on fluorescence induction, does appreciably decrease photosynthetic O_2 evolution in bundle sheath strands [12], and clearly can supply electrons to poise adequately the cyclic electron flow essential for the fixation of CO_2 in the bundle sheath.

Conclusions

Mature maize bundle sheath cells contain very little PS II and such as does exist is unlikely to be of importance in vivo. PS I-driven cyclic electron flow is the major electron-transfer pathway in bundle sheath chloroplasts, producing the ATP required for CO_2 fixation. Malate is the principal redox poising agent for activation of cyclic electron flow, with a small contribution from dihydroxyacetone phosphate; both these compounds are produced in vivo in the mesophyll chloroplast and exported to the bundle sheath. It is possible that the oxygen tension in the bundle sheath compartment is lower than that in the surrounding tissues, a feature which would accelerate the rates of cyclic electron flow and, possibly, CO_2 fixation.

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References

1 Hatch, M.D. and Osmond, C.B. (1976) in Encyclopedia of Plant Physiology, New Series, Vol. 3 (Stocking, C.R. and Heber, U., eds.), pp. 144–184, Springer-Verlag, Heidelberg
2 Woo, K.C., Anderson, J.M., Boardman, N.K., Downton, W.J.S., Osmond, C.B. and Thorne, S.W. (1970) Proc. Natl. Acad. Sci. U.S.A. 67, 18–25
3 Anderson, J.M., Boardman, N.K. and Spencer, D. (1971) Biochim. Biophys. Acta 245, 253–258
4 Anderson, K.S., Bain, J.M., Bishop, D.G. and Smillie, R.M. (1972) Plant Physiol. 49, 461–466
5 Smillie, R.M., Anderson, K.S., Tobin, N.F., Entsch, B. and Bishop, D.G. (1972) Plant Physiol. 49, 471–475
6 Ku, S.B., Gutierrez, M., Kanai, R. and Edwards, G.E. (1973) Z. Pflanzenphysiol. 72, 320–337
7 Bazzaz, M.B. and Govindjee (1973) Plant Physiol. 52, 257–262
8 Usuda, H., Kanai,. R. and Miyachi, S. (1975) Plant Cell Physiol. 16, 485–494
9 Hardt, H. and Kok, B. (1978) Plant Physiol. 62, 59–63
10 Walker, G.H. and Izawa, S. (1979) Plant Physiol. 63, 133–138
11 Hatch, M.D. and Kagawa, T. (1976) Arch. Biochem. Biophys. 175, 39–53
12 Chapman, K.S.R., Berry, J.A. and Hatch, M.D. (1980) Arch. Biochem. Biophys. 202, 330–341
13 Leegood, R.C., Crowther, D., Walker, D.A. and Hind, G. (1981) FEBS Lett. 126, 89–92
14 Arnon, D.I. and Chain, R.K. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 4961–4965
15 Arnon, D.I. and Chain, R.K. (1977) Plant Cell Physiol., Special Issue on Photosynthetic Organelles, pp. 129–147
16 Slovacek, R.E. and Hind, G. (1977) Plant Physiol. 60, 538–542
17 Hind, G., Mills, J.D. and Slovacek, R.E. (1978) in Proceedings of the 4th International Congress on Photosynthesis, Reading, (Hall, D.O., Coombs, J. and Goodwin, R.E., eds.), pp. 591–600, The Biochemical Society, London
18 Crowther, D., Mills, J.D. and Hind, G. (1979) FEBS Lett. 98, 386–380
19 Mills, J.D., Crowther, D., Slovacek, R.E., Hind, G. and McCarty, R.E. (1979) Biochim. Biophys. Acta 547, 127–137
20 Mills, J.D., Mitchell, P. and Barber, J. (1976) Photobiochem. Photobiophys. 1, 3–9
21 Crowther, D. and Hind, G. (1980) Arch. Biochem. Biophys. 204, 568–577
22 Arnon, D.I. (1949) Plant Physiol. 24, 1–15
23 Witt, H.T. (1971) Q. Rev. Biophys. 4, 365–477
24 Bouges-Bocquet, B. (1982) in Function of Quinones in Energy Conserving Systems (Trumpower, B.L., ed.), Academic Press, New York, in the press
25 Egneus, H., Heber, U., Mathiesen, U. and Kirk, M. (1975) Biochim. Biophys. Acta 408, 252–268
26 Leegood, R.C. and Walker, D.A. (1981) Arch. Biochem. Biophys. 212, 644–650
27 Elstner, E.F. and Heupel, A. (1974) Z. Naturforsch. 29c, 594–571
28 Crowther, D., Leegood, R.C., Walker, D.A. and Hind, G. (1983) Biochim. Biophys. Acta 722, 127–136
29 Allen, J.F. (1975) Nature 256, 559–600
30 Harel, E., Lea, P.J. and Miflin, B.J. (1977) Planta 134, 195–200
31 Slack, C.R., Hatch, M.D. and Goodchild, D.J. (1969) Biochem. J. 114, 489–498
32 Rathnam, C.K.M. and Edwards, G.E. (1975) Arch. Biochem. Biophys. 171, 214–225
33 Trebst, A. (1980) Methods Enzymol. 69, 675–715
34 Chapman, K.S.R. and Hatch, M.D. (1981) Aust. J. Plant Physiol. 8, 237–248

35 Papageorgiou, G. (1975) in Bioenergetics of Photosynthesis (Govindjee, ed.), pp. 319–371, Academic Press, New York
36 Malkin, S. and Kok, B. (1966) Biochim. Biophys. Acta 126, 413–432
37 Golbeck, J.H., Martin, I.F., Velthuys, B.R. and Radmer, R. (1981) in Proceedings of the 5th International Congress on Photosynthesis (Akoyunoglou, G., ed.), Vol. 5, pp. 533–546. Balaban International Science Services, Philadelphia
38 Gregory, R.P.F., Droppa, M., Horvath, G. and Evans, E.H. (1979) Biochem. J. 180, 253–256
39 Mayne, B.C., Dee, A.M. and Edwards, G.E. (1974) Z. Pflanzenphysiol. 74, 275–291
40 Farineau, J. (1975) Physiol. Plant 33, 300–309
41 Farineau, J. (1975) Physiol. Plant 33, 310–315
42 Rathnam, C.K.M. and Edwards, G.E. (1977) Arch. Biochem. Biophys. 182, 1–13
43 Roux, E. and Faludi-Daniel, A. (1977) Biochim. Biophys. Acta 461, 25–30
44 Horvath, G., Droppa, M., Mustardy, L.A. and Faludi-Daniel, A. (1978) Planta 141, 239–244
45 Slovacek, R.E., Mills, J.D. and Hind, G. (1978) FEBS Lett. 87, 73–76
46 Heber, U., Egneus, H., Hanck, U., Jensen, M. and Köster, S. (1978) Planta 143, 41–49
47 Ziem-Hanck, U. and Heber, U. (1980) Biochim. Biophys. Acta 591, 266–274
48 Raven, J.A. (1977) Curr. Adv. Plant Sci. 9, 579–590
49 Osmond, C.B. and Smith, F.A. (1976) in Intercellular Communication in Plants: Studies on Plasmodesmata (Gunning, B.E.S. and Robards, A.W., eds.), pp. 229–241, Springer-Verlag, Heidelberg
50 Johnson, H.S. and Hatch, M.D. (1970) Biochem. J. 119, 273–280
51 Asami, S., Inoue, K. and Akazawa, T. (1979) Arch. Biochem. Biophys. 196, 581–587
52 Huber, S.C., Kanai, R. and Edwards, G.E. (1973) Planta 113, 53–66
53 Usuda, H. and Miyachi, S. (1977) Plant Cell Physiol. 18, 1109–1120
54 Takahama, U., Shimizu-Takahama, J. and Heber, U. (1981) Biochim. Biophys. Acta 637, 530–539
55 Robinson, S.P. and Walker, D.A. (1979) Biochim. Biophys. Acta 545, 528–536
56 Laing, W.A., Stitt, N.M. and Heldt, H.W. (1981) Biochim. Biophys. Acta 637, 348–359