Light-driven uptake of pyruvate into mesophyll chloroplasts from maize

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(i) Photosynthesis in maize, a C_4 species, involves a movement of pyruvate from the bundle sheath to the mesophyll, which has been suggested to occur by symplastic diffusion. (ii) Measurement of metabolite distribution in maize leaves revealed that the bulk of the pyruvate in the mesophyll was not in diffusion equilibrium with pyruvate in the bundle sheath, indicating that most of the mesophyll pyruvate was not located in the cytosol but accummulated in a subcellular compartment. (iii) In concurrence with these results, transport measurements revealed a light-dependent active transport of pyruvate into maize mesophyll chloroplasts, probably driven by a proton gradient. This active transport of pyruvate may be regarded as an important factor in driving a malate-pyruvate shuttle in C_4 photosynthesis.

Pyruvate transport *Chloroplast envelope* *C_4 metabolism* *Photosynthesis* *Maize*

1. INTRODUCTION

C_4 plants, such as maize or sugar cane, can grow rapidly in high light by having a device for concentrating CO_2 at the site of photosynthetic CO_2 fixation. For this, the CO_2 from the air is trapped in the mesophyll cells in contact with the air space by the PEP carboxylase reaction yielding oxaloacetate. In maize, a malic enzyme type C_4 plant, this oxaloacetate is reduced in the maize mesophyll chloroplasts to malate, which then passes to the bundle sheath cells. There it is decarboxylated by malic enzyme, providing CO_2 for the Calvin cycle in the bundle sheath chloroplasts. Pyruvate, the other decarboxylation product, passes back to the mesophyll cells, where it is converted to PEP by the pyruvate phosphate dikinase located in the mesophyll chloroplasts (fig.1) [1]. Such an accumulation of CO_2 in the bundle sheath cells, caused by different CO_2 equilibria of carboxylation and decarboxylation reactions, requires that these two processes are spatially separated to minimize diffusion of the CO_2 back to the mesophyll cells.

The flow of metabolites between the mesophyll and bundle sheath cells has been postulated to occur via diffusion through plasmodesmata connecting these cells [1,2]. Theoretical considerations showed, that the necessary diffusion fluxes of

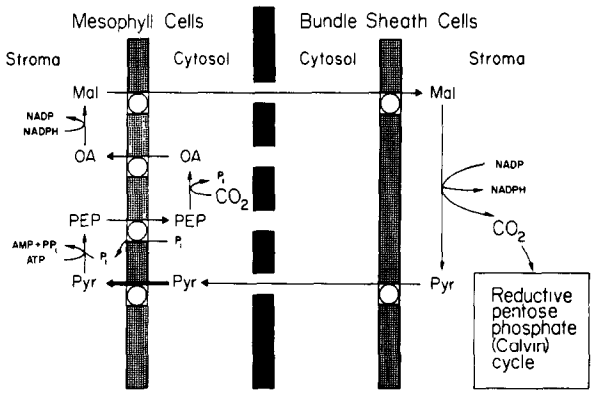

Fig.1. Metabolic scheme of C_4 metabolism in maize.

Abbreviations: PEP, phospho*enol*pyruvate; Pyr, pyruvate; pCMS, *p*-chloromercuribenzenesulfonate; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone

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malate and pyruvate across the distance between the mesophyll and bundle sheath cells required a concentration gradient of these substances in the range 10^{-3}–10^{-2} M [2]. The recent development of methods for assaying the distribution of metabolites between mesophyll and bundle sheath cells of maize leaves [3–5] opened the possibility of investigating whether such concentration gradients actually existed.

It will be shown here that in maize leaves a concentration gradient exists not only for malate but also for pyruvate. In addition to this, an active transport of pyruvate into the mesophyll chloroplasts is involved in driving the flux of pyruvate from the bundle sheath to the mesophyll chloroplasts.

2. MATERIALS AND METHODS

Maize (*Zea mays* var. Vorlo) was grown in a glasshouse (24°C day, 15°C night) with 14 h/day supplemented lighting. Fully grown leaves were taken from 4–6 week old plants and chloroplasts were prepared as described in [6]. Transport of pyruvate was measured by silicone oil filtering centrifugation [7] in a medium containing 0.33 M sorbitol and 50 mM Hepes-KOH (pH 7.8). Chloroplasts (0.05–0.15 mg Chl/ml) were incubated for 8 min in the light before starting uptake by addition of [^{14}C]pyruvate (Amersham Buchler, Braunschweig, FRG; spec. act., 1 Ci/mol). Transport was terminated by centrifugation for 30 s in a Beckman Microfuge.

For measuring the conversion of pyruvate to PEP by intact chloroplasts, 900 μl chloroplasts (0.04 mg chl/ml) were incubated in a medium containing 0.33 M sorbitol, 50 mM Hepes-KOH (pH 7.8) and 5 mM KH_2PO_4, when indicated, for 8 min in the light or dark. The reaction was started with pyruvate and stopped after 5, 10 and 15 s by the addition of 15 μl 70% $HClO_4$. Formation of PEP was assayed as in [8,9].

The intracellular distribution of pyruvate in maize leaves was measured as in [5]. Maize leaves were detached, held in the dark for 2 h and illuminated for 25 min in air or CO_2-free air provided by passage of air in a closed system through a $Ca(OH)_2$ column. Water-saturated air was used in all cases. Material was subsequently quenched in liquid nitrogen, fractionated and assayed as in [5].

3. RESULTS AND DISCUSSION

3.1. *Intercellular distribution of malate and pyruvate in illuminated maize leaves*

For measuring the distribution of metabolites between the mesophyll and bundle sheath cells, maize leaves were partially homogenized under liquid nitrogen to produce particles which are enriched in bundle sheath or mesophyll, and separated by filtering them through a series of nylon nets under liquid nitrogen [5]. From assay of metabolites and marker enzymes in the separated fractions, the metabolite content of mesophyll and bundle sheath cells was calculated. Overall concentrations in the two cell types were estimated by assuming that chlorophyll is equally distributed between the mesophyll and bundle sheath cells, and that the combined stroma and cytosol in each cell type is 40 μl/mg chl. Although these values are only approximations, they provide an estimate of cellular concentrations in vivo.

The intercellular distribution of malate and pyruvate in illuminated leaves under ambient CO_2 (table 1) has been observed before [4,5]. Whereas for malate an apparent concentration difference between the mesophyll and bundle sheath cells of 18 mM is found, a corresponding gradient of pyruvate into the opposite direction is not observed, the pyruvate in the mesophyll being even slightly higher than in the bundle sheath cells. The existing concentration gradients of diffusing C_4 metabolites should collapse when the supply of CO_2 is interrupted. In the absence of CO_2, the concentration difference of malate between the mesophyll and bundle sheath cells indeed de-

Table 1

Intercellular distribution of malate and pyruvate in illuminated maize leaves in the presence and absence of ambient CO_2

	Malate		Pyruvate	
	Meso-phyll	Bundle sheath	Meso-phyll	Bundle sheath
+ CO_2	35	17	6	5
− CO_2	17	12	3	0.5

For details see section 2 and text. Values are expressed as mM

creased to less than one third of the corresponding value in ambient CO_2. This result, and similar data on the distribution of dihydroxyacetone phosphate and 3-phosphoglycerate in the presence and absence of CO_2 (submitted), may be regarded as experimental verification of the earlier hypothesis [1,2] that in C_4 metabolism the corresponding metabolite fluxes between the mesophyll and bundle sheath cells occur by symplastic diffusion. Since such symplastic diffusion pathways are not supposed to be specific for small molecules, pyruvate would be expected to follow this diffusion pathway as well, and there should therefore be a concentration gradient of pyruvate, too. Such a gradient is not apparent, however, since in the presence of CO_2 an almost equal distribution of the pyruvate is observed. In the absence of CO_2, the pyruvate in the bundle sheath cells is drastically reduced, as one might have expected, but the pyruvate in the mesophyll remains high. Obviously, this pyruvate cannot be in diffusion equilibrium with the pyruvate in the bundle sheath cells. These results suggest that the bulk of the pyruvate in the mesophyll cells is not located in the cytosol, but accumulated in a subcellular compartment by active transport.

3.2. *Transport of pyruvate into isolated maize mesophyll chloroplasts*

It was therefore necessary to identify the intracellular compartment that accumulates pyruvate. Chloroplasts were a likely candidate, as the pyruvate phosphate dikinase is chloroplastic. Huber and Edwards [10] had shown earlier, that mesophyll chloroplasts from the C_4 plant *Digitaria sanguinalis* contained a pyruvate carrier, but they studied this transport only in the dark, where the concentrations of pyruvate in the chloroplasts were always much less than in the medium. We therefore measured the uptake of [^{14}C]pyruvate into isolated maize mesophyll chloroplasts by silicone layer filtering centrifugation, to see whether these chloroplasts were capable of active transport of pyruvate, and how this was energized.

To measure the uptake of [^{14}C]pyruvate into mesophyll chloroplasts, it had to be ensured that the accumulation of the ^{14}C radioactivity in the chloroplasts represented an accumulation of the pyruvate, and not of conversion products from it. The first conversion product of pyruvate formed within the chloroplasts is PEP, catalyzed by the action of pyruvate phosphate dikinase. To measure this conversion, we added pyruvate to intact mesophyll chloroplasts under different conditions and assayed the initial rates of PEP formation as described in section 2. The rate of pyruvate conversion to PEP in the absence of P_i at 4°C was negligible in comparison to the measured rate of [^{14}C]pyruvate uptake into the chloroplasts, and even at 20°C was relatively low (table 2). Thus, in the absence of P_i and especially at low temperature, the accumulation of the label from [^{14}C]pyruvate in the sorbitol-impermeable space of maize mesophyll chloroplasts does indeed represent an accumulation of pyruvate in the stroma.

Table 2

Rates of the uptake of [^{14}C]pyruvate into maize mesophyll chloroplasts, and of the conversion of pyruvate into PEP

Conditions	[^{14}C]Pyr uptake (4°C)	PEP formation	
		4°C	20°C
Dark,			
1 mM Pyr	1.4	0	0.07
1 mM Pyr + 5 mM P_i		0.45	1.1
Light,			
1 mM Pyr	9.6	0.05	0.7
1 mM Pyr + 5 mM P_i		2.5	58.6

Values are expressed as μmol/mg chl per h

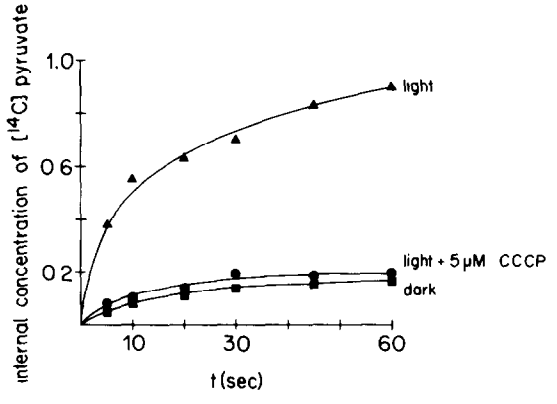

Fig.2. Transport of [^{14}C]pyruvate (0.2 mM) into the sorbitol-impermeable space of maize mesophyll chloroplasts. Temp. 4°C.

The kinetics of the uptake of pyruvate into mesophyll chloroplasts are shown in fig.2. In the light, pyruvate was taken up so rapidly that the kinetics could only be resolved at 4°C, and after 1 min the concentration of pyruvate inside exceeded the external concentration (0.2 mM) by a factor of about 5. In the dark, however, the rate of transport was much lower than in the light, and the internal concentration reached a concentration equivalent to that of the surrounding medium. The protonophore CCCP, known to abolish the light-dependent proton gradient across the chloroplast envelope [11], had similar effects as darkness. In concurrence with the results of Huber and Edwards [10], we found an inhibition of pyruvate transport by the sulfhydryl reagent pCMS (fig.3).

To characterize the effect of light on the pyruvate transport, we measured the concentration dependence of this transport in the dark and in the light. A double reciprocal plot of the data revealed that light did not affect the apparent affinity of the pyruvate transport towards its substrate. The apparent K_m value (0.8–0.9 mM) was rather similar to the corresponding value (0.6–1.0 mM) observed by Huber and Edwards [10] in mesophyll chloroplasts from *D. sanguinalis*. The V_{max} for pyruvate transport in the light (35 μmol/mg chl per h (4°C)) was more than 7 times higher than in the dark (4.5 μmol/mg chl per h (4°C)), showing that the stimulation of pyruvate transport in the light was due to an increase of V_{max}. Note that in chloroplasts from the C_3 plant spinach, the transport of pyruvate is not observed [10].

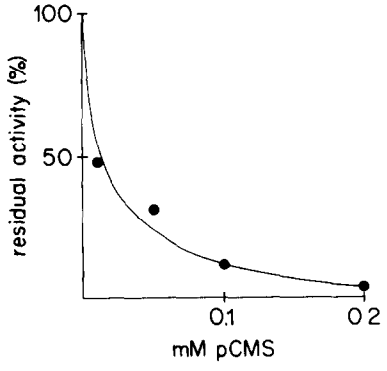

Fig.3. Inhibition of the transport of [^{14}C]pyruvate (0.2 mM) into maize mesophyll chloroplasts in the light by pCMS. The uninhibited rate was 5.6 μmol/mg chl per h. Temp. 4°C.

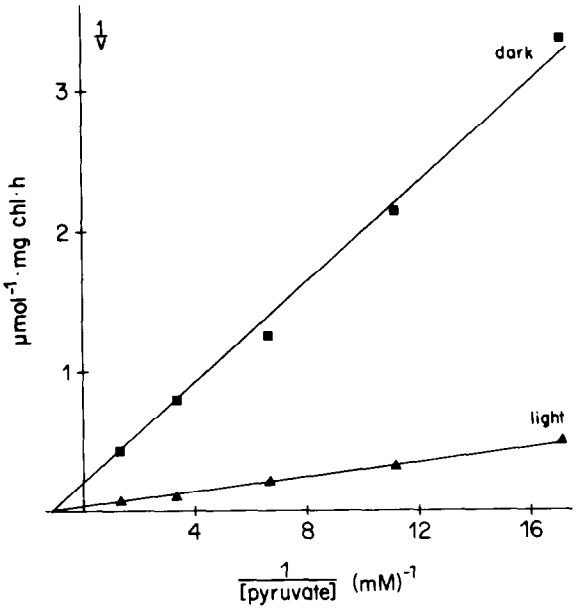

Fig.4. Concentration dependence of the transport of [^{14}C]pyruvate into maize mesophyll chloroplasts. Temp. 4°C.

As mentioned before, pyruvate transport into maize mesophyll chloroplasts at 20°C is too fast to be resolved by silicone layer filtration experiments. To obtain a minimal estimate of the transport rate at 20°C, we measured the conversion of pyruvate to PEP by intact chloroplasts with added P_i in the light, and in this way obtained a V_{max} of 95 μmol/mg chl per h, which is of the same order of magnitude as the carbon flux in C_4 photosynthesis.

4. CONCLUSION

Our results have demonstrated that maize mesophyll chloroplasts possess a pyruvate translocator, which by light-dependent active transport, probably driven by a proton gradient across the envelope, enables the accumulation of pyruvate in the chloroplast stroma. This finding offers an explanation for the earlier notion (table 1) that the bulk of the pyruvate in the mesophyll cells is accumulated in a subcellular compartment apart from the cytosol. The chloroplasts show this ability to accumulate pyruvate. The properties of pyruvate transport into chloroplasts are such, that in illuminated leaves the cytosolic pyruvate con-

centrations in the mesophyll are expected to be very low. Thus it appears most likely that a concentration gradient for pyruvate between the bundle sheath and the mesophyll cytosol also exists, supporting the earlier hypothesis [1] that pyruvate traverses the distance between the bundle sheath and mesophyll cytosol by diffusion. The light-driven pumping of pyruvate into the maize mesophyll chloroplasts may be regarded as an additional factor in driving the malate-pyruvate shuttle of C_4 photosynthesis.

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