Starch degradation in chloroplasts isolated from C_3 or CAM (crassulacean acid metabolism)-induced *Mesembryanthemum crystallinum* L.

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C_3 or crassulacean acid metabolism (CAM)-induced *Mesembryanthemum crystallinum* plants perform nocturnal starch degradation which is linear with time. To analyse the composition of metabolites released by isolated leaf chloroplasts during starch degradation we developed a protocol for the purification of starch-containing plastids. Isolated chloroplasts from C_3 or CAM-induced *M. crystallinum* plants are also able to degrade starch. With respect to the endogenous starch content of isolated plastids the rate of starch degradation in these organelles is close to the observed rates of starch degradation in intact leaves. The combined presence of P_i, ATP, and oxaloacetate is identified to be the most positive effector combination to induce starch mobilization. The metabolic flux through the oxidative pentose-phosphate pathway in chloroplasts isolated from CAM-induced *M. crystallinum* is less than 3.5 % compared with other metabolic routes of starch degradation. Here we report that starch-degrading chloroplasts isolated from CAM-induced *M. crystallinum* plants use exogenously supplied oxaloacetate for the synthesis of malate. The main products of starch degradation exported into the incubation medium by these chloroplasts are glucose 6-phosphate, 3-phosphoglyceric acid, dihydroxyacetone phosphate and glucose. The identification of glucose 6-phosphate as an important metabolite released during starch degradation is in contrast to the observations made on all other types of plastids analysed so far, including chloroplasts isolated from *M. crystallinum* in the C_3 state. Therefore, we analysed the transport properties of isolated chloroplasts from *M. crystallinum*. Surprisingly, both types of chloroplasts, isolated from either C_3 or CAM-induced plants, are able to transport glucose 6-phosphate in counter exchange with endogenous P_i, indicating the presence of a glucose 6-phosphate translocator as recently demonstrated to occur in other types of plastids. The composition of metabolites released and the stimulatory effect of oxaloacetate on the rate of starch degradation are discussed with respect to the acidification observed for CAM leaves during the night.

INTRODUCTION

The diurnal turnover of transitory starch is an important feature of plant metabolism. Disturbances of starch metabolism induce significant changes in both plant growth rate and plant habit [1,2]. During the day, photosynthetic CO_2 is fixed and converted into sugar phosphates which are partly used for the synthesis of transitory starch inside the plastids [3]. This starch is degraded during the following night period [4] and supplies, after conversion of the mobilization products into sucrose, the heterotrophic plant tissues with carbohydrates.

Up to now there have been few experimental data available about the nature of the intermediates released by leaf chloroplasts during the process of starch degradation. Stitt and ap Rees [5] have shown that isolated chloroplasts from pea leaves are able to mobilize carbon formerly bound in starch. The identification of phosphorylated intermediates as the main products of starch degradation was confirmed for isolated spinach chloroplasts [6]. From these phosphorylated intermediates plastid-type-specific amounts are allocated into the oxidative pentose-phosphate pathway (OPP) [5,6].

In addition, there are further indications that different types of chloroplasts use specific metabolic routes for starch degradation. Isolated spinach chloroplasts release, in contrast to pea chloroplasts, substantial amounts of glucose and maltose during starch degradation [6]. Moreover, ap Rees and colleagues recently demonstrated that chloroplasts of an *Arabidopsis thaliana* mutant which were unable to degrade starch at appreciable rates lack a 40 kDa chloroplast-envelope protein supposed to be the plastidic glucose transporter [7]. As the absence of this envelope protein correlates with strongly reduced rates of starch degradation and increased stromal glucose concentrations it can be assumed that chloroplasts of wild-type *A. thaliana* release mainly neutral sugars during the process of starch degradation.

So far, there are no data available concerning the composition of the metabolites of starch degradation in isolated chloroplasts of CAM (crassulacean acid metabolism) plants. However, the mobilization of starch in these plants is as important as in C_3 plants since carbon, formerly bound in starch, is used for the synthesis of phosphoenolpyruvate (PEP), acting as the nocturnal acceptor for CO_2 [8]. Recently, it has been demonstrated that the induction of CAM in *Mesembryanthemum crystallinum* leads to a dramatic increase in the activity of starch-degrading enzymes and to increased rates of starch turnover [9]. These observations underline the important function of starch degradation in malic-enzyme-type CAM plants, known to use transitory starch as a carbon source for nocturnal PEP synthesis.

The aim of this study was to identify the products of starch degradation in isolated chloroplasts from C_3 or CAM-induced *M. crystallinum*. In addition, we were interested in the identi-

Abbreviations used: CAM, crassulacean acid metabolism; DHAP, dihydroxyacetone phosphate; Glc6P, glucose 6-phosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NADP-GAPDH, NADP-dependent GAPDH; MDH, malate dehydrogenase; OAA, oxaloacetate; OPP, oxidative pentose-phosphate pathway; PEP, phosphoenolpyruvate; PFK, phosphofructose kinase; PGA, 3-phosphoglyceric acid.

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fication of effectors of starch degradation which might be involved in CAM.

MATERIALS AND METHODS

Growth of *M. crystallinum* and induction of CAM

Growth of *M. crystallinum* was conducted hydroponically as described in [10]. Plants were grown in a climate chamber with 10 h day-length and illuminated with white light (Philips Sont-Agro, Philips, Osnabrück, Germany) at an irradiance of 200 μmol quanta/m^2 per s. To induce CAM the NaCl concentration in the hydroponic solution of 8-week-old plants was successively increased to 500 mM by the addition of 100 mM NaCl per 24 h for 5 days. Plants were grown for five further days under these conditions and used for the indicated experiments within 1 week. To determine that this treatment induces CAM we quantified the amount of free organic acids in leaf-tissue extracts in the morning and in the evening. CAM-induced *M. crystallinum* contain 76.3 μmol of H^+ equivalents/mg of chlorophyll at the end of the night period, representing four times more than at the end of the light period (19.0 μmol of H^+ equivalents/mg of chlorophyll). In contrast, C_3 plants which were not treated with NaCl did not show any diurnal acid changes (results not shown). In addition, the increase in the activity of PEP carboxylase (5-fold) after NaCl treatment indicated that CAM was induced in a pronounced manner (results not shown).

Isolation of starch-containing chloroplasts from *M. crystallinum*

It is known that the isolation of starch-containing chloroplasts is hampered by the high specific density of starch granules leading to the disruption of the plastid envelope during purification. Recently, we have developed a protocol allowing the isolation of starch-rich chloroplasts from glucose-fed spinach leaves [11]. This protocol is used here with the indicated modifications for the enrichment of starch-containing chloroplasts from *M. crystallinum*.

All steps of the isolation were carried out on ice at 0–4 °C. Leaves of *M. crystallinum* were harvested immediately after the onset of the dark period, cut into small pieces (5 mm^2), and homogenized for 3 × 3 s in a 1 litre beaker of a Waring Blendor (Waring Corporation, Hartford, CT, U.S.A.) in 120 ml of medium A consisting of: 450 mM mannitol, 30 mM Hepes (pH 7.8, NaOH), 2 mM EDTA and 0.5 % BSA (acetone washed). The homogenate was filtered through three layers of muslin and one layer of miracloth (Calbiochem, CA, U.S.A.) and the filtrate was subsequently centrifuged for 2 min at 2000 ***g*** in a Sorvall SS34 rotor (Sorvall, Dreieich, Germany). The sediment was resuspended in 1–2 ml of medium A. To this chloroplast suspension, 50 ml of medium A containing in addition 35 % Percoll (Pharmacia, Freiburg, Germany) was added, mixed, and divided between two 25 ml tubes. This preparation was centrifuged for 25 min at 50000 ***g*** in an ultracentrifuge (Kontron, München, Germany; equipped with a Sorvall T 1250 rotor). The chloroplast band, which appeared close to the bottom of the tube, was removed with a plastic Pasteur pipette and the fractions were pooled and diluted with 50 ml of medium A. This suspension was divided between two tubes and centrifuged for 90 s at 3500 ***g***. The resulting chloroplast sediment was resuspended in 1 ml of chloroplast medium consisting of: 450 mM mannitol, 30 mM Hepes (pH 7.8, NaOH), 2 mM EDTA, 1 mM $MnCl_2$, 1 mM $MgCl_2$, and 0.2 % BSA (acetone washed). The chloroplasts were stored on ice and used within 1 h of preparation. Chlorophyll was quantified according to the method of Arnon [12].

Determination of intactness and contamination with cellular components of isolated chloroplasts from *M. crystallinum*

Chloroplast purity was estimated by measuring marker-enzyme activities specific for cellular compartments in both crude extracts and purified chloroplasts. NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (NADP-GAPDH, EC 1.2.1.13) was assayed as a chloroplast marker according to the method of Holtum and Winter [13]; UDP glucose pyrophosphorylase (UDPGlcPPase, EC 2.7.7.9; which is known to be a cytosolic marker enzyme) according to the method of Bergmeyer [14]; and citrate synthase (EC 4.1.3.7) and α-mannosidase (EC 3.2.1.24), as mitochondrial and vacuolar marker enzymes respectively, according to the method of Stitt et al. [15]. All enzyme activity assays were checked to be linear with time and increasing amounts of sample.

The intactness of the chloroplast preparations from CAM-induced *M. crystallinum* were estimated by measuring the latent activity [16] of the chloroplastic marker enzyme NADP-GAPDH in an intact sample (addition of 450 mM mannitol to the measuring buffer) and in a lysed sample in which the plastids had been disintegrated by ultrasonication (3 × 3 s) prior to measurement.

Incubation of starch-containing chloroplasts from *M. crystallinum* to promote the release of carbon formerly bound in starch

Starch-containing chloroplasts from *M. crystallinum* were incubated in the dark at room temperature for given periods of time in chloroplast medium containing various effectors. Chloroplasts, equivalent to 50 μg of chlorophyll, were incubated in a final volume of 300 μl. The metabolism was stopped by addition of 30 μl of 'killing mixture' consisting of 60 % $HClO_4$, 0.5 % Triton X-100 and 10 mM EDTA, and subsequently stored for 15 min on ice. The samples were afterwards neutralized by addition of 50–60 μl of 1 M KOH/5 M triethanolamine and insoluble perchlorate was removed by centrifugation for 5 min at 16000 ***g*** in an Eppendorf centrifuge. The clear supernatant was transferred into a new reaction vessel and stored at −80 °C until required for use.

Quantification of released metabolites and recoveries

Quantification of the indicated metabolites was perfomed according to the methods described in [17] using a highly sensitive dual-wavelength spectrophotometer (Sigma-Eppendorf ZFP 22, Eppendorf, Hamburg, Germany) at 334 nm (365 nm for malate determination) and 405 nm as the reference wavelength. The reliability of the extraction and the metabolite measurements was checked by recovery experiments in which representative amounts of each metabolite were added to the 'killing mixture' prior to the extraction.

Quantification of starch in leaf tissues or isolated chloroplasts from *M. crystallinum*

To measure the starch content in leaf tissues or isolated chloroplasts from *M. crystallinum* starch was hydrolytically digested [18] and the soluble glucose subsequently quantified according to the method described in [17]. To extract starch from *M. crystallinum*, leaf tissue samples of 1 cm diameter were collected and immediately frozen in liquid nitrogen. The plant tissue was subsequently ground in a precooled mortar (−190 °C) and quantitatively transferred into an Eppendorf reaction vessel. A 1 ml aliquot of 80 % ethanol was added and the suspension was

vigorously mixed for 2 min. To sediment undissolvable material the samples were centrifuged for 5 min at 16000 ***g*** and the supernatant was transferred into new reaction vessels and used for chlorophyll quantification.

The residual sediment was suspended in 300 μl of 0.1 M sodium acetate (pH 4.7) medium and autoclaved for 2 h at 120 °C. After cooling, 150 μl of sodium acetate medium, containing 5 units each of α-amylase and amyloglucosidase, was added and starch was quantitatively digested for 2 h at 37 °C in an Eppendorf thermomixer.

Radioactive labelling of newly synthesized starch and quantification of the metabolic flux through the OPP in isolated chloroplasts from CAM-induced *M. crystallinum*

To quantify the involvement of the OPP during starch degradation in isolated chloroplasts from CAM-induced *M. crystallinum* we labelled newly synthesized starch using [U-^{14}C]glucose 6-phosphate (Glc6P) and measured subsequently the release of $^{14}CO_2$.

To label newly synthesized starch we incubated chloroplasts isolated from CAM-induced *M. crystallinum* equivalent to 0.3 mg of chlorophyll/ml in chloroplast medium containing in addition: 5 mM $KHCO_3$, 0.1 mM KH_2PO_4, 1000 units/ml catalase and 1 mM [U-^{14}C]Glc6P (3 MBq/mmol; ICN, Meckenheim, Germany). Incubation was carried out at room temperature under continuous illumination for 30 min [19]. To remove unincorporated radioactivity, plastids were sedimented for 2 min in a precooled Eppendorf centrifuge (2 °C) at 5700 ***g*** and the sediment subsequently resuspended in 1 ml of ice-cold chloroplast medium. This procedure was repeated twice. Finally, plastids were resuspended in chloroplast medium yielding a chlorophyll concentration of 1 mg/ml, and were kept on ice until use.

$^{14}CO_2$ release from radioactively labelled starch was quantified by collecting $^{14}CO_2$ in KOH [20]. Chloroplasts, equivalent to 75 μg of chlorophyll, were incubated in chloroplast medium in a 2.0 ml Eppendorf reaction vessel (final volume 300 μl) in darkness. Released $^{14}CO_2$ was collected in 50 μl of 1 M KOH in the excised tip of a 400 μl Eppendorf reaction vessel fixed with laboratory grease to the inner wall. The incubation was stopped by adding 100 μl of 'stop solution' consisting of 50 % ethanol/0.1 M HCl to the sample. The 'stop solution' was added through the closed lid of the Eppendorf reaction vessel using a syringe. The remaining hole was sealed with laboratory grease. After acidification, the vessels were kept closed for 16 h at room temperature. The radioactivity trapped in the KOH solution was quantified by liquid scintillation counting using a Packard Tricarb 2500 scintillation counter (Packard, Darmstadt, Germany).

Transport of P_i across the envelope of isolated plastids in the counter-exchange mode

Isolated plastids from various origins export endogenous P_i at highest rates solely in counter exchange with exogenous transport substrates [21,22]. Therefore, we have preloaded chloroplasts from *M. crystallinum* with [^{32}P]P_i and used these subsequently to measure the rates of P_i transport in the counter-exchange mode. Preloading of plastids was performed in a manner similar to the method of Fliege et al. [21] by incubating isolated chloroplasts (1 mg of chlorophyll/ml) in 2.5 ml Eppendorf reaction vessels for 60 min on ice in chloroplast medium (see above) containing in addition 10 mM $KH_2{}^{32}PO_4$ (specific radioactivity 1–3 MBq/mmol; NEN Du-Pont, Dreieich, Germany). To remove chloroplast medium containing external radioactivity, plastids were sedimented in an Eppendorf centrifuge (2 °C, 2 min, 5700 ***g***). The sediment was carefully resuspended in 1 ml of ice-cold chloroplast medium and subsequently centrifuged as described above. This wash step was repeated twice and the final plastid preparation was resuspended in chloroplast medium to a chlorophyll concentration of 1 mg/ml. The latent activities of NADP-GAPDH in washed chloroplasts did not differ substantially ($\pm$3 %, $n = 4$) from those of freshly isolated organelles. It has been shown previously that starchless or starch-rich plastids from various tissues are suited for this treatment [11,20,21].

Plastids preloaded with P_i were used to study the short-term transport kinetics by using the silicone-oil filtration method [23] with the modifications as described in [18]. This method enables the quantitative separation of isolated plastids from the incubation medium by centrifugation. To start export of [^{32}P]P_i, unlabelled P_i, dihydroxyacetone phosphate (DHAP), or Glc6P were added at a concentration of 200 μM. The incubations were carried out for 15 s at 10 °C. The rates of [^{32}P]P_i export were linear with increasing amounts of plastids and with time for 20 to 25 s (results not shown). Termination of the transport process was achieved by separation of the plastids from the incubation medium by centrifugation of the samples at 16000 ***g*** through silicone oil (Wacker AP 100, Wacker-Chemie, München, Germany) in a Microfuge E (Beckman, Hannover, Germany). Radioactivity appearing in the supernatant was quantified by beta counting.

RESULTS

Characterization of isolated chloroplasts

As given in Table 1 about one-fifth of the starch-rich chloroplasts present in the first homogenate are recovered at the end of the purification. Isolated chloroplasts from C_3 or CAM-induced *M. crystallinum* are virtually free of detectable levels of vacuolar components. The cytosolic contamination is below 0.2 % (S.E.M. 0.1 %, $n = 4$). In relation to mitochondria the isolated chloroplasts are enriched 5-fold leading to a 21.6 % (CAM) and 19.5 % (C_3) (S.E.M. 2.0 %, $n = 4$) contamination with citrate synthase activity.

To study the metabolism of an organelle it is desired to isolate it in an intact state. According to the latency of NADP-GAPDH isolated chloroplasts from C_3 or CAM-induced *M. crystallinum* exhibit routinely an intactness of 76 and 72 %, respectively (S.E. M. 3 %, $n = 4$). This degree of intactness is even higher than the intactness of starch-less chloroplasts from CAM-induced *M.*

Table 1 Activities of marker enzymes in the homogenate and purified chloroplasts from C_3 or CAM-induced *M. crystallinum*

The activities of the indicated marker enymes are measured according to the methods given in the Materials and methods section. Abbreviation: n.d., not detectable.

	Enzyme activity (μmol/min)					
	Homogenate		Isolated chloroplasts		Recovery (%)	
Enzyme	C_3	CAM	C_3	CAM	C_3	CAM
NADP-GAPDH	187.2	239.4	41.2	45.5	22.0	19.0
UDPGlc-PPase	132.8	148.3	0.07	0.06	> 0.05	> 0.05
Citrate synthase	6.2	7.1	0.27	0.29	4.3	4.1
α-Mannosidase	8.2	9.8	n.d.	n.d.	n.d.	n.d.

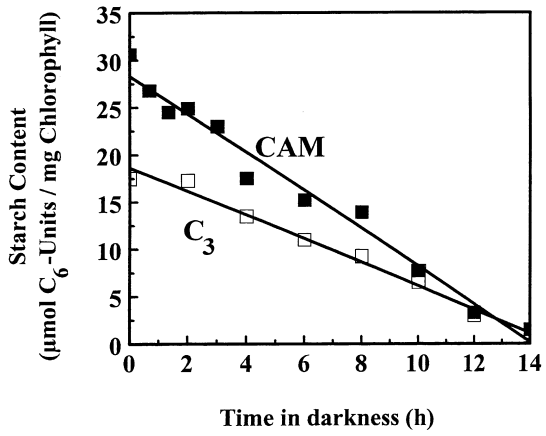


Figure 1 Starch degradation in leaves of CAM-induced and uninduced *M. crystallinum*

Harvesting of samples and quantification of starch was performed as described in the Materials and methods section. Data represent the means of six samples taken from three plants. Standard errors were always less than 7%.

crystallinum isolated using a different protocol [9]. These values of intactness were also confirmed using the ferricyanide method (results not shown).

Rate of starch degradation in intact leaves and starch content in chloroplasts purified from C_3 or CAM-induced *M. crystallinum*

To compare the rates of starch degradation in isolated chloroplasts from *M. crystallinum* with the corresponding rates of starch degradation in the intact tissue we first measured the decrease of leaf starch content during the night period. As shown in Figure 1, at the end of the light period leaves of CAM-induced *M. crystallinum* contain starch equivalent to 30 μmol of glucose (C_6) units/mg of chlorophyll. Immediately after onset of the dark period starch degradation is apparent and proceeds linearly with time during the entire night (Figure 1). After 14 h in the dark the starch content is decreased to 1.5 μmol of C_6 units/mg of chlorophyll. Therefore, about 7.1 % of the leaf starch is degraded per hour. The leaf-starch content of plants in the C_3 state at the end of the light period amounts to 18 μmol of C_6 units/mg of chlorophyll (Figure 1). Similar to starch degradation in CAM-induced plants, C_3 plants degrade transitory starch over the entire night leading to 0.9 μmol of C_6 units/mg of chlorophyll at the end of the dark period (Figure 1).

It is known that the isolation of starch-containing chloroplasts from various tissues is hampered by the high specific density of starch granules [24]. A method involving isopycnic Percoll centrifugation has recently allowed the purification of starch-rich chloroplasts from glucose-fed spinach leaves [11]. Using that protocol with the given modifications we were able to routinely purify chloroplasts from C_3 or CAM-induced *M. crystallinum* which both contained starch equivalent to 14.2 μmol of C_6 units/mg of chlorophyll (S.E.M. 1.5, $n = 5$). In the case of chloroplasts from C_3 plants this value is close to the starch content of the intact leaf (Figure 1), whereas starch in chloroplasts isolated from CAM-induced plants is about 50 % of the leaf-starch content (Figure 1). Attempts to purify chloroplasts from CAM-induced plants with higher endogenous starch contents have been unsuccessful so far (results not shown). However, Stitt and Heldt [6] also used chloroplasts containing about 50 % of the leaf-starch content for a successful characterization of starch degradation in that type of plastid. We will show here that starch-containing chloroplasts isolated from CAM-induced *M. crystallinum* represent a useful system for the characterization of starch degradation. This conclusion is based on the following observations: (i) starch degradation in isolated chloroplasts occurs linearly with time for at least 1 h; (ii) starch degradation in isolated chloroplasts is strictly dependent upon the intactness of the organelle; and (iii) in relation to the endogenous starch content of isolated chloroplasts the rate of starch mobilization is similar to the rate of starch mobilization in intact leaves.

Composition of metabolites released by isolated chloroplasts from CAM-induced or C_3 *M. crystallinum*

Prior to the quantification of metabolites released we performed recovery experiments for each starch mobilization compound. Recoveries were as follows: Glc6P, 89.5 %; DHAP, 89.7 %; 3-phosphoglyceric acid (PGA), 93.3 %; glucose, 96.7 %; sucrose, 89.6 %; maltose, 108.4 %; malate, 99.4 %. Standard errors were in all cases less than 3 % ($n = 5$). The data presented below are corrected for these values. Control experiments demonstrate that more than 97 % of all metabolites synthesized during starch degradation are exported into the incubation medium, allowing starch degradation to proceed for at least 1 h (see below).

Isolated plastids from either pea or spinach leaves [5,6], or cauliflower buds [20] are able to release carbon formerly bound in starch. Obviously, the same holds true for isolated chloroplasts from CAM-induced *M. crystallinum* as the incubation of these plastids in darkness leads to an increase of the metabolite concentration in the incubation medium. After 20 min of incubation in the dark the carbon content of the incubation medium is increased by around 110 nmol of C_6/mg of chlorophyll (Table 2) which is mainly due to export of glucose. We also measured maltose and sucrose in the samples. The presence of sucrose in isolated plastids of various origins has already been documented [6,20]. Since the content of both metabolites did not show any changes in all conditions tested the results are not shown. As the levels of both metabolites remained unchanged it is indicated that maltose is not liberated during starch degradation and the appearance of other intermediates is not due to conversion of sucrose.

The additional presence of P_i (5 mM) or ATP (2 mM) leads to a stimulation of the rate of starch degradation due to increased synthesis of Glc6P and DHAP (Table 2). Neither fructose 6-phosphate nor glucose 1-phosphate could be detected in the medium. Interestingly, oxaloacetate (OAA) applied at a concentration of 0.2 mM also induces a stimulatory effect on the rate of starch mobilization. In contrast to the application of P_i or ATP the OAA-stimulated rate of starch degradation is due to substantially increased rates of PGA export (Table 2).

The presence of both P_i and ATP induces the highest rates of starch mobilization, leading to an increase in the sum of released C_6 units to about 750 nmol/mg of chlorophyll per h (Table 2). As the average starch content of isolated chloroplasts from CAM-induced *M. crystallinum* is 14.2 μmol of C_6 units/mg of chlorophyll an increase in the sum of C_6 units in the incubation medium to 750 nmol/mg of chlorophyll per h corresponds to a rate of starch degradation of approx. 5.3 % per h, which is similar to the rate of starch degradation in the intact leaf (Figure 1). It should be noted here, that starch degradation in chloroplasts isolated from CAM-induced *M. crystallinum* is dependent upon the intactness of the organelles (see below) and that the intactness of the plastid preparations was about 72 % (see above). This might be the reason why these chloroplast preparations do not mobilize

Table 2 Release of metabolites by chloroplasts isolated from CAM-induced *M. crystallinum* during starch degradation

Isolated chloroplasts were incubated for 20 min (control 0 min) in chloroplast medium containing the given effectors in addition. Effector concentrations used were: P_i, 5 mM; ATP, 2 mM; OAA, 0.2 mM; and Triton X-100, 0.5%. Stoppage of metabolism and quantification of the metabolites were carried out as described in the Materials and methods section. Data are the means of three independent samples. Standard errors were always less then 8%. For determination of the rate of metabolite release (ΣC_6/h) the data were corrected for the metabolite amounts present in the chloroplasts at the beginning of the incubation (0 min control). Values in parentheses are *P* values.

	Metabolites					
	(nmol/mg of chlorophyll)					(nmol/mg of chlorophyll per h)
Effector(s)	DHAP	PGA	Glc6P	Glc	ΣC_6	ΣC_6
None (control)	24.0	7.2	56.0	46.5	118.1	
None	51.2	22.0	87.8 (0.025)	103.1	227.5 (0.025)	328.2
P_i	45.6	31.2	120.8 (0.025)	77.3	236.5 (0.025)	355.2
ATP	92.2	15.6	145.9 (0.025)	77.3	277.1 (0.025)	477.0
OAA	24.9	159.4	97.8 (0.025)	117.7	307.7 (0.025)	568.8
P_i + OAA	42.9	67.9	166.0 (0.025)	88.4	309.8 (0.025)	575.1
P_i + ATP	85.8	17.6	212.4 (0.025)	103.8	367.9 (0.025)	749.4
P_i + ATP + OAA	65.1	58.7	216.2 (0.025)	85.0	363.1 (0.025)	735.0
P_i + ATP + OAA + Triton X-100	31.9	8.4	102.0 (0.025)	66.9	189.1 (0.025)	213.0

Table 3 Release of metabolites by chloroplasts isolated from C_3 *M. crystallinum* during starch degradation

Isolated chloroplasts were incubated for 20 min (control 0 min) in chloroplast medium containing the given effectors in addition. Effector concentrations used were: P_i, 5 mM; ATP, 2 mM; OAA, 0.2 mM; and Triton X-100, 0.5%. Stoppage of metabolism and quantification of the metabolites were carried out as described in the Materials and methods section. Data are the means of three independent samples. Standard errors were always less then 8%. For determination of the rate of metabolite release (ΣC_6/h) the data were corrected for the metabolite amounts present in the chloroplasts at the beginning of the incubation (0 min control). Values in parentheses are *P* values.

	Metabolites					
	(nmol/mg of chorophyll)					(nmol/mg of chlorophyll per h)
Effector(s)	DHAP	PGA	Glc6P	Glc	Maltose	ΣC_6
None (control)	18.4	24.8	18.0	44.2	55.2	
None	18.4	27.2	18.4 (0.025)	79.2	75.6	232.2
P_i	78.6	40.7	18.4 (0.025)	70.4	55.0	192.9
ATP	117.5	25.6	50.6 (0.025)	66.5	156.2	920.7
OAA	11.4	142.6	13.8 (0.025)	97.7	138.6	814.5
P_i + OAA	76.6	171.8	21.3 (0.025)	126.5	180.9	1318.8
P_i + ATP	224.2	36.0	53.1 (0.025)	88.8	159.2	1188.6
P_i + ATP + OAA	117.7	241.7	51.4 (0.025)	98.4	179.8	1484.7
P_i + ATP + OAA + Triton X-100	39.8	28.0	42.2 (0.025)	24.6	94.1	284.1

endogenous starch with exactly the same rate as observed in the intact leaf (7.1% per h).

To determine whether the observed rates of starch degradation are dependent upon the physiological intactness of the plastids we disrupted the plastid envelope by the addition of Triton X-100. This treatment reduces the rate of starch mobilization significantly (Tables 2 and 3), demonstrating that this process depends strictly upon metabolism within the intact plastid rather than upon hydrolytic activities occurring on starch granules which are not surrounded by an intact envelope. The addition of NAD and NADP to the incubation medium did not further stimulate the release of metabolites from starch in chloroplasts isolated from *M. crystallinum* (results not shown). This observation indicates that neither the OPP nor glycolysis are limited by the availability of oxidized pyridine nucleotides which might have leaked out during the process of plastid isolation.

To gain insight into the features of metabolism specific for CAM we have carried out similar experiments with plastids from C_3 *M. crystallinum* (Table 3). On the basis of C_6 units, the rate of starch degradation in C_3 plastids is about twice as high compared with the rate of starch degradation in chloroplasts isolated from CAM-induced *M. crystallinum* (Table 3). This difference might be due to a substantial osmotic stress affecting chloroplasts isolated from CAM-induced *M. crystallinum*. Indeed, it has been observed already that the process of photosynthetic CO_2 fixation in isolated chloroplasts from CAM-induced *M. crystallinum* is significantly lower than in corresponding chloroplasts isolated from C_3 plants [25]. Nevertheless, comparing the effects of the various effectors on the release of metabolites indicates some similarities of starch degradation in both types of chloroplasts. For example, the rate of starch degradation in both plastid types is substantially increased by the exogenous application of ATP or OAA (Tables 2 and 3).

However, there are also substantial differences in the mobilization of starch in these two types of chloroplasts. For example the release of maltose or Glc6P as the major export product during starch mobilization appears to be restricted to a specific type of plastid. While chloroplasts isolated from CAM-induced plants mainly export Glc6P, the main export product of plastids from C_3 *M. crystallinum* is maltose (Tables 2 and 3). This

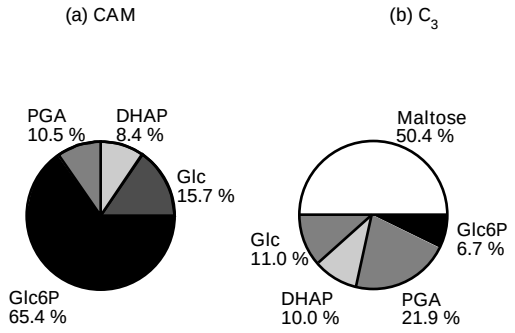


Figure 2 Composition of the starch degradation products of isolated chloroplasts from CAM-induced (a) or C_3 (b) *M. crystallinum*

Shown is the metabolite composition (sum of C_6 units) appearing in the medium in the presence of P_i (5 mM), ATP (2 mM), and OAA (0.2 mM). Data are taken from Tables 2 and 3.

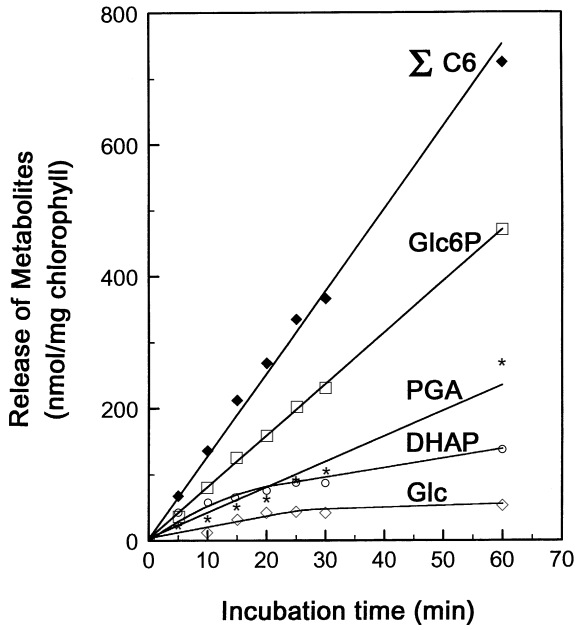


Figure 3 Time course of metabolite release by chloroplasts isolated from CAM-induced *M. crystallinum*

Isolated chloroplasts were incubated for the given periods of time in chloroplast medium containing in addition: P_i 5 mM, ATP 2 mM, OAA 0.2 mM. Stoppage of metabolism and quantification of the metabolites were carried out as described in the Materials and methods section. The data are corrected for the metabolite content at 0 min of incubation. Data are the means of three independent samples. Standard errors were always less then 8%.

compound is not synthesized by plastids from CAM-induced *M. crystallinum* (Figure 2), whereas in contrast, chloroplasts from C_3 plants hardly export Glc6P (Figure 2).

To characterize further the process of starch degradation in isolated chloroplasts from CAM-induced *M. crystallinum* plants, we analysed the time dependency of the carbon release. Obviously, starch degradation occurs linearly with time for at least 1 h (Figure 3), demonstrating that the time interval used in the other experiments (20 min of incubation) allows for the calculation of maximal rates. The same time-dependency was also found for starch degradation in chloroplasts isolated from C_3 plants (results not shown).

Short-term transport across the envelope of chloroplasts isolated from CAM-induced *M. crystallinum*

The observation that isolated starch-containing chloroplasts from CAM-induced *M. crystallinum* release significant amounts of Glc6P into the incubation medium is in contrast to the observations made for isolated chloroplasts from C_3 *M. crystallinum*. This discrepancy might, at least partly, be due to different transport properties of the chloroplasts examined. Therefore, we analysed the transport properties of isolated chloroplasts from C_3 or CAM-induced *M. crystallinum* by employing the back-exchange method using plastids preloaded with $[^{32}P]P_i$. The quantity of radioactivity appearing in the incubation medium after addition of unlabelled potential counter-exchange substrates to the chloroplast suspension thus indicated metabolites sharing the same transport protein.

As known for spinach chloroplasts [21] these plastids transport P_i or DHAP with high rates across the plastid envelope (Table 4) whereas the exogenous application of Glc6P does not promote significant rates of $[^{32}P]P_i$ export (Table 4). However, isolated chloroplasts from CAM-induced *M. crystallinum* exhibit different transport properties. In addition to the transport of P_i or DHAP they exhibit substantial rates of $[^{32}P]P_i$ export induced by the presence of Glc6P in the incubation medium (Table 4). The rate of Glc6P-induced $[^{32}P]P_i$ export reaches approx. 12% of the rate of $[^{32}P]P_i$ export observed after addition of unlabelled P_i to the incubation medium. Interestingly, chloroplasts isolated from C_3 plants showed similar transport capacities concerning the rate of Glc6P transport (Table 4). However, the relative rate of DHAP transport across the envelope of C_3 chloroplasts is substantially lower than in the case of chloroplasts isolated from CAM-induced plants (Table 4).

Malate synthesis by chloroplasts isolated from CAM-induced *M. crystallinum*

As documented by the data in Table 2 the addition of OAA to the incubation medium induces a significant stimulation of the rate of starch degradation. However, since malate might be a potential product of OAA metabolism and as malic acid plays an important role in CAM plants we measured changes in the malate content under different incubation conditions.

Isolated chloroplasts from CAM-induced *M. crystallinum* contain approx. 95 nmol of malate/mg of chlorophyll (Table 5). This level remains almost unchanged up to 20 min after incubation in chloroplast medium (Table 5). The presence of 0.2 mM OAA in the incubation medium induces a rate of malate synthesis of 120 nmol/mg of chlorophyll per 20 min (Table 5). This rate of OAA-dependent malate synthesis would lead to an accumulation of approx. 10 μmol of H^+ equivalents/mg of chlorophyll during a 14 h dark period, which corresponds to 17.5% of the total H^+ equivalents nocturnally synthesized in the leaf tissue from which the chloroplasts were purified (see above). The additional presence of P_i reduces the rate of OAA-dependent malate synthesis to 45 nmol/mg of chlorophyll per 20 min (Table 5).

Metabolic flux through the OPP

Isolated chloroplasts from pea or spinach leaves are able to use carbon formerly bound in starch to drive the OPP. However, according to the results shown in Table 6 the flux through the OPP in isolated chloroplasts from CAM-induced *M. crystallinum* plants is low, reaching only about 3.5% of the sum of C_6 units mobilized (Table 2). Neither the effectors, P_i and ATP, nor the

Table 4 Transport capacities of chloroplasts isolated from spinach leaves or leaves of *M. crystallinum*

Isolated chloroplasts were preloaded with [^{32}P]P_i as described in the Materials and methods section. After removal of exogenous radioactivity the export of [^{32}P]P_i was initated by the addition of the given effectors (each effector was given at a 0.2 mM concentration). Data are the mean of three independent experiments. Standard errors were always less than 10%.

Origin of plastids	Effector	Rate of [^{32}P]P_i back exchange (μmol/mg of chlorophyll per h)	[^{32}P]P_i back exchange (% of P_i rate)
Spinach leaves*	P_i	45.7	100.0
	DHAP	46.6	102.0
	Glc6P	0.0	0.0
M. crystallinum CAM	P_i	16.1	100.0
	DHAP	16.6	103.1
	Glc6P	1.9	11.8
M. crystallinum C_3	P_i	17.8	100.0
	DHAP	13.0	73.0
	Glc6P	2.4	13.5

* Data are taken from [36].

Table 5 Malate synthesis in chloroplasts isolated from CAM-induced *M. crystallinum*

Isolated chloroplasts were incubated for 20 min (none, control = 0 min) in chloroplast medium containing the given effectors in addition. Effector concentrations used were: OAA, 0.2 mM; and P_i, 5 mM. Quenching of metabolism and quantification of malate was performed as described in the Materials and methods section. Data represent the means of three independent samples. Standard errors were always less than 8%.

Effector(s)	Malate content (nmol/mg of chlorophyll)
None (control)	95.0
None	106.2
OAA	214.0
OAA + P_i	140.8

Table 6 Metabolic flux through the OPP in chloroplasts isolated from CAM-induced *M. crystallinum*

Radioactive labelling of newly synthesized starch and quantification of $^{14}CO_2$ release was carried out as described in the Materials and methods section. Effector concentrations used were: P_i, 5 mM; ATP, 2 mM; OAA, 0.2 mM; and Triton X-100, 0.5%. Data represent the means of three independent samples. Standard errors were always less than 8%.

Effectors	Metabolic flux (nmol of C_6 units/mg of chlorophyll per h)
P_i + ATP	19.8
P_i + ATP + OAA	25.2
P_i + ATP + OAA + Triton X-100	2.4

additional presence of OAA induced higher fluxes through the OPP (Table 6).

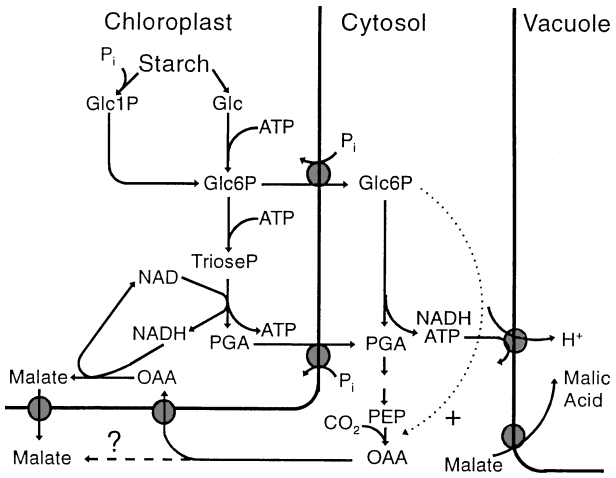
To measure the rate of metabolic flux through the plastidic OPP it is necessary to label starch in isolated chloroplasts prior to the quantification of the flux through the OPP. This labelling is achieved by incubation of illuminated chloroplasts in the presence of [U-^{14}C]Glc6P. Unexpectedly, however, we were not able to label starch in isolated chloroplasts from C_3 plants and we were not able to identify experimental conditions allowing for such labelling.

DISCUSSION

The degradation of transitory starch is an important feature of leaf chloroplasts. In CAM-induced plants belonging to the malic-enzyme type the metabolic products of nocturnal starch degradation are mainly converted into the primary CO_2 acceptor PEP in the cytosol [8]. Because of the important role of starch metabolism in CAM plants it is not suprising that starch turnover increases during CAM induction (Figure 1). However, until now there have been no experimental data available concerning the composition of metabolites released from starch-degrading chloroplasts of any CAM plant.

The results presented in Table 2 demonstrate that isolated chloroplasts purified from CAM-induced *M. crystallinum* are able to release carbon formerly bound in starch at a rate of about 330 nmol of C_6 units/mg of chlorophyll per h. Under the chosen conditions starch is mainly degraded to glucose which is exported into the incubation medium. The export of glucose into the incubation medium in the absence of additional P_i can be explained on the basis of the enzymic equipment of *M. crystallinum* chloroplasts. Paul et al. [9] demonstrated that the induction of CAM in *M. crystallinum* is accompanied by a dramatic increase in the activity of a chloroplastic α-amylase. It is remarkable, that the addition of both P_i and ATP stimulates the rate of starch degradation about 2.3-fold (Table 2). This observation is in agreement with the stimulatory effect of ATP and P_i on the rate of starch degradation in isolated amyloplasts from cauliflower buds [20].

Here we demonstrate that the addition of OAA leads to a stimulation of the rate of starch degradation in isolated chloroplasts (Table 2). OAA is converted into malate (Table 5) via a plastidic malate dehydrogenase (MDH) requiring reduced pyridine dinucleotides. For the following reasons we would like to suggest that nocturnal malate synthesis is catalysed by a plastidic NAD-dependent MDH: (i) the plastidic NADP-dependent MDH is known to be redox-modulated and inactive in darkness [26,27]; (ii) chloroplasts from CAM-induced *M. crystallinum* plants possess an NAD-dependent MDH [10] (in addition, this enzyme activity was also demonstrated in various plastids of heterotrophic origin [28]); (iii) the synthesis of malate by isolated chloroplasts leads to a nearly stoichiometric synthesis of PGA (Tables 2 and 5). It is known that the use of NAD as coenzyme for the GAPDH reaction is independent from the reductive activation of this enzyme [29]. Thus, this enzyme is able to



Scheme 1 Metabolic fluxes during starch degradation in mesophyll cells of CAM-induced *M. crystallinum*

catalyse nocturnal NADH synthesis leading to the generation of ATP and PGA via the coupled phosphoglycerate kinase reaction.

We propose that the stimulatory effect of OAA on the rate of starch mobilization is most likely due to stromal synthesis of ATP connected to the synthesis of PGA (Table 2, Scheme 1). This assumption is reinforced by the observation that exogenous ATP acts as a strong activator upon the rate of starch degradation (Tables 2 and 3), indicating that the rate of internally synthesized ATP is higher than the rate of ATP uptake. This assumption concurs with low rates of ATP uptake demonstrated to occur across the envelope of chloroplasts, namely chloroplasts from spinach mesophyll cells [30]. The transport of ATP is mediated by a specific ATP/ADP transporter which has been cloned and sequenced recently [31].

The OAA-stimulated degradation of starch cannot be further stimulated by the additional application of exogenous P_i (Table 2). This effect is most likely due to an increased export of Glc6P (Tables 2 and 4) in the presence of the counter-exchange compound P_i. This assumption received further support through the analysis of the effect of P_i on the rate of OAA-dependent malate synthesis (Table 5). Obviously, due to an increased export of Glc6P less carbon can be oxidized via plastidic glycolysis and concomitantly fewer reduced pyridine dinucleotides are synthesized; this leads to reduced rates of malate synthesis (Table 5).

It is remarkable that the OAA-stimulated starch degradation correlates with increased amounts of phosphorylated intermediates released (Tables 2 and 3). This observation indicates that isolated chloroplasts from CAM-induced *M. crystallinum* contain substantial quantitites of P_i, making it difficult to distinguish the contribution of amylolytic and phosphorylytic starch degradation activities. However, the composition of metabolites released by chloroplasts incubated in chloroplast medium without additional effectors indicates for the following reasons that both metabolic pathways of starch degradation occur: (i) the neutral sugar glucose is exported from the chloroplasts; and (ii) the sum of Glc6P and DHAP liberated during 20 min of incubation would require the synthesis of about 60 nmol of ATP/mg of chlorophyll (Table 2) to drive the hexokinase and phosphofructokinase reactions involved in the synthesis of these metabolites. As the PGA content in the incubation medium increases only by 15 nmol/mg of chlorophyll there would not be sufficient metabolic flux through the phosphoglycerate kinase reaction to catalyse the synthesis of ATP. This calculation can be justified since the metabolic flux through the OPP, which would also lead to the synthesis of DHAP, is low (Table 6).

To our knowledge, we have identified the first plastid type that releases Glc6P (Table 2). Chloroplasts isolated from leaves of pea, spinach, or C_3 *M. crystallinum* do not transport substantial amounts of hexose phosphates during starch degradation [5,6] (Figure 2). The export of Glc6P by chloroplasts isolated from CAM-induced *M. crystallinum* indicates that these plastids possess an envelope protein mediating Glc6P transport. The results given in Table 4 clearly demonstrate that Glc6P is transported in counter exchange with P_i which is in accordance with the observations about Glc6P transport across the envelope of plastids isolated from pea roots [32] or cauliflower buds [22]. Chloroplasts isolated from spinach leaves hardly transport Glc6P across the envelope [21] (Table 4), whereas chloroplasts localized in specific plant tissues possess the ability to transport Glc6P. Recently, we have demonstrated that chloroplasts purified from green pepper fruits are able to use exogenous Glc6P for starch biosynthesis [19] and that chloroplasts purified from glucose-fed spinach leaves develop the ability to transport Glc6P at high rates [11].

The fact that chloroplasts isolated from *M. crystallinum* in the C_3 state show similar rates of short-term Glc6P transport to chloroplasts from CAM-induced plants (Table 4) suggests that the ability for Glc6P transport is not a consequence of CAM induction. However, until now we have not been able to explain why chloroplasts isolated from CAM-induced *M. crystallinum* export Glc6P, whereas chloroplasts from C_3 plants do not. One explanation for this discrepancy is that the stromal phosphofructose kinase (PFK) is more active in CAM than in C_3 plants. This activation most likely occurs by an allosteric mechanism as it is shown that induction of CAM in *M. crystallinum* does not induce higher activity of extractable PFK [13]. Activated PFK would lead to lowered concentrations of hexose phosphates and thus prevent export of Glc6P. Interestingly, plastids isolated from cauliflower buds which also possess a highly active Glc6P transporter [22] do not export Glc6P under conditions of starch degradation [20], indicating that specific metabolic conditions in the stroma are responsible for the composition of metabolites released.

The major function of nocturnal starch degradation in malic enzyme CAM plants is to supply the cytosol with carbon skeletons needed for the synthesis of PEP, which acts as the primary CO_2 acceptor during the night [8,33]. However, the cellular site of OAA reduction during nocturnal acidification of CAM leaf tissues is unknown. According to the data presented above, at least one-fifth of the total amount of free acids can be synthesized in chloroplasts during the night. In the case where not all free organic acids are represented by malic acid this percentage might be even higher.

With respect to the composition of metabolites released during starch degradation in chloroplasts purified from CAM-induced *M. crystallinum* and to the effect of OAA on both the rate of starch degradation and malate synthesis, we would like to draw the following picture of interaction between starch degradation and CAM (Scheme 1): during starch degradation chloroplasts release mainly Glc6P into the cytosol. This hexose phosphate fulfils two major functions in CAM: (i) Glc6P acts as a carbon precursor for the cytosolic synthesis of PEP; and (ii) Glc6P was identified to be an allosteric activator of PEP carboxylase in all plant species analysed so far [34]. Therefore, in addition to the nocturnal activation of PEP carboxylase in CAM plant tissues by phosphorylation [35] the allosteric activator Glc6P is present in

the cytosol (Table 2). The increased activity of PEP carboxylase leads to the synthesis of OAA, which is afterwards imported into the plastids for conversion into malate (Table 5). This observation is remarkable since the addition of OAA leads to an increased rate of starch degradation which could provide carbon skeletons for cytosolic PEP synthesis (Table 2). Furthermore, the release of Glc6P during starch degradation in chloroplasts from CAM-induced *M. crystallinum* leads to an indirect transfer of ATP since during the cytosolic conversion of Glc6P into PEP one ATP molecule is liberated which can be used in CAM leaf tissues to energize the uptake of malic acid into the vacuole.

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