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Respiration of Sugars in Spinach (*Spinacia oleracea*), Maize (*Zea mays*), and *Chlamydomonas reinhardtii* F-60 Chloroplasts with Emphasis on the Hexose Kinases[1]

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The role of hexokinase in carbohydrate degradation in isolated, intact chloroplasts was evaluated. This was accomplished by monitoring the evolution of $^{14}CO_2$ from darkened spinach (*Spinacia oleracea*), maize (*Zea mays*) mesophyll, and *Chlamydomonas reinhardtii* chloroplasts externally supplied with ^{14}C-labeled fructose, glucose, mannose, galactose, maltose, and ribose. Glucose and ribose were the preferred substrates with the *Chlamydomonas* and maize chloroplasts, respectively. The rate of CO_2 release from fructose was about twice that from glucose in the spinach chloroplast. Externally supplied ATP stimulated the rate of CO_2 release. The pH optimum for CO_2 release was 7.5 with ribose and fructose and 8.5 with glucose as substrates. Probing the outer membrane polypeptides of the intact spinach chloroplast with two proteases, trypsin and thermolysin, decreased $^{14}CO_2$ release from glucose about 50% but had little effect when fructose was the substrate. Tryptic digestion decreased CO_2 release from glucose in the *Chlamydomonas* chloroplast about 70%. $^{14}CO_2$ evolution from [1-^{14}C]-glucose-6-phosphate in both chloroplasts was unaffected by treatment with trypsin. Enzymic analysis of the supernatant (stroma) of the lysed spinach chloroplast indicated a hexokinase active primarily with fructose but with some affinity for glucose. The pellet (membranal fraction) contained a hexokinase utilizing both glucose and fructose but with considerably less total activity than the stromal enzyme. Treatment with trypsin and thermolysin eliminated more than 50% of the glucokinase activity but had little effect on fructokinase activity in the spinach chloroplast. Tryptic digestion of the *Chlamydomonas* chloroplast resulted in a loss of about 90% of glucokinase activity.

Saltman (1953), the first to investigate hexokinase in higher plants, observed that at least 20% of the total hexokinase activity in leaves was in the particulate cell fraction, the rest being in the cytosol. Resolution of the total hexokinase activity revealed four kinases in spinach leaves: two hexokinases active with Glc, Fru, and Man and two fructokinases functioning primarily with Fru but to a far lesser extent with Glc and Man (Baldus et al., 1981; Schnarrenberger, 1990). Essentially similar results have been described for pea seed (Copeland et al., 1978; Turner and Copeland, 1981), germinating maize seeds (Dohlert, 1989), and castor beans (Miernyk and Dennis, 1983).

The bulk of the particulate hexokinase was bound to the mitochondria (Saltman, 1953), but there have been reports of hexokinase and fructokinase associated with plastids. Thus, a fructokinase is a constituent of the spinach chloroplast (Schnarrenberger, 1990) but Stitt et al. (1978) found that 90% of a glucokinase associated only with the external surface of the outer membranes of the pea chloroplast envelope. In contrast, the plastids in developing castor bean endosperm tissue host a hexokinase with broad substrate specificity in the stroma (Miernyk and Dennis, 1983). The aim of the work described in this paper was to study the hexokinases in whole chloroplasts isolated from spinach, mesophyll tissue of maize, and the green alga *Chlamydomonas reinhardtii* F-60.

Our experimental approach was to monitor the release of $^{14}CO_2$ in darkened chloroplast preparations externally supplied with ^{14}C-labeled sugars: Glc, Fru, Man, Gal, maltose, and Rib. Inasmuch as gluconate-6-P dehydrogenase in the oxidative pentose-P pathway is solely responsible for CO_2 release in these chloroplasts (Ahluwalia et al., 1989; Chen and Gibbs, 1991), a positive release would indicate an enzymic profile including a sugar kinase reaction and the eventual formation of Glc-6-P, the entry substrate into the pathway.

To determine the level of association between the kinases and the intact chloroplasts of *C. reinhardtii* and spinach, we utilized trypsin and thermolysin, two relatively nonspecific proteases, as probes, because their effects are primarily directed to enzymes located on the outer envelope membrane of the chloroplast (Joyard et al., 1983; Cline et al., 1984). Following proteolytic treatment of the intact chloroplasts, we measured residual kinase activity as well as $^{14}CO_2$ release from the ^{14}C-labeled sugars to demonstrate whether the kinase reactions are located within the stroma or on the cytosolic side of the chloroplast.

MATERIALS AND METHODS

Plant Material and Preparation of Intact and Lysed Chloroplasts

Spinach (*Spinacia oleracea* L.) var Longstanding Bloomdale was grown and used to prepare intact chloroplasts as described earlier (Kow et al., 1977).

Chlamydomonas reinhardtii mutant strain F-60 (obtained

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from Professor R.K. Togasaki, Indiana University, Bloomington) was grown under fluorescent light on an acetate-supplemented medium as described previously (Willeford and Gibbs, 1989). The procedure for isolating intact chloroplasts from F-60 followed that of Klein et al. (1983). The mutant is unable to fix CO_2, because it lacks ribulose-5-kinase.

Corn (*Zea mays* var Golden Cross) was grown in vermiculite and fertilized once a week with Hoagland solution in a controlled environment with 12 h light (25°C) and 12 h darkness (15°C). Intact mesophyll chloroplasts were isolated from 2- to 3-week-old plants by the procedure of Jenkins and Russ (1984).

Lysed chloroplasts were obtained by thoroughly homogenizing intact chloroplasts in water and then making this suspension up to the composition of the final resuspension medium used for intact chloroplasts.

$^{14}CO_2$ Measurements

Rates of CO_2 evolution were determined by adding intact chloroplasts of spinach and maize to a reaction mixture containing 50 mM Hepes-NaOH (pH 7.5), 330 mM sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM MgATP, and ^{14}C-sugars other than Glc. In the case of spinach, when Glc was the substrate the buffer was 50 mM Tris-HCl (pH 8.5). For F-60, intact chloroplasts were added to a reaction mixture containing 50 mM Tris-HCl (pH 8.2), 120 mM mannitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM MgATP, and ^{14}C-sugar.

The reaction mixtures were incubated at 25°C for 10 min in the main compartments of darkened 15-mL Warburg flasks carrying the chloroplast suspension in a sidearm and 0.3 mL of 2 M triethanolamine in the center well. The vessels were sealed with serum stoppers, and the reactions were initiated by tipping in the chloroplasts from the sidearm to bring the final reaction volume to 1.0 mL. Following termination of the reaction by addition of perchloric acid to a final concentration of 5% (v/v), the flasks were shaken slowly for an additional hour to allow the released $^{14}CO_2$ to be absorbed totally by the ethanolamine. The triethanolamine was transferred into a scintillation vial containing SML biodegradable counting scintillant. Radioactivity was determined in a Beckman L5-150 liquid scintillation counter.

Enzyme Assays

Hexokinase was assayed by coupling the production of NADPH in the presence of an excess of Glc-6-P dehydrogenase according to the method of Turner and Copeland (1981) with slight modifications. The reaction mixture contained in a total volume of 1 mL was 25 mM Tris-HCl (pH 8.2); 7 mM $MgCl_2$; 5 mM ATP; 0.33 mM NADP; 1 unit of Glc-6-P dehydrogenase; 5 mM Glc, 5 mM Fru, or 5 mM Man; 0.1% Triton X-100; and intact chloroplasts containing approximately 15 to 20 μg of Chl. When Fru was the substrate, 2 units of phosphoglucose isomerase were added. When Man was the substrate, 2 units each of phosphoglucose isomerase and phosphomannose isomerase were included.

To assay Glc-6-P dehydrogenase and gluconate-6-P dehydrogenase, Glc was replaced by 5 mM Glc-6-P or 5 mM gluconate-6-P.

The rate of formation of NADPH was measured at 25°C in a Gilford recording spectrophotometer (model 250) by monitoring the A_{340}.

Treatment with Trypsin and Thermolysin

Trypsin treatment was carried out in a reaction mixture containing 50 mM Hepes-NaOH (pH 7.5), 120 mM mannitol for *Chlamydomonas* and 330 mM sorbitol for spinach, 1 mM $MgCl_2$ 1 mM $MnCl_2$, and 2 mM EDTA. Trypsin (0.67 mg mL^{-1}, final concentration) or, where indicated, trypsin plus trypsin inhibitor (2 mg ml^{-1}, final concentration) was added to an ice-cold suspension of intact chloroplasts (15–30 μg of Chl mL^{-1}), which was incubated at 25°C for 30 min. Because trypsin was dissolved in 1 mM HCl, the same amount of HCl was added to the suspension lacking trypsin. At the end of the incubation, trypsin inhibitor (2 mg mL^{-1}, final concentration) was added to the sample that had been treated with trypsin alone. The chloroplast suspensions were used directly for measurement of $^{14}CO_2$ release or for enzymic analysis.

Thermolysin treatment was carried out in a reaction mixture similar to that used for trypsin. Thermolysin (300 μg mL^{-1}, final concentration) and 0.5 mM $CaCl_2$ or thermolysin (300 μg mL^{-1}), 0.5 mM $CaCl_2$, and 7 mM EGTA (inhibitor by removing calcium) were added to an ice-cold suspension of intact chloroplasts (20–50 μg of Chl mL^{-1}), which was then incubated at 30°C for 30 min. At the end of the incubation, 7 mM EGTA was added to the samples that contained thermolysin and $CaCl_2$ alone. The chloroplast suspensions were used directly for measurement of $^{14}CO_2$ release or for enzymic analysis.

Chl Determination

Chl was determined by the method of Arnon (1949).

Reagents

[U-^{14}C]Glc (3.3 mCi $mmol^{-1}$), [U-^{14}C]Fru (4.9 mCi $mmol^{-1}$), [1-^{14}C]Man (4.5 mCi $mmol^{-1}$), [1-^{14}C]Gal (3.1 mCi $mmol^{-1}$), [U-^{14}C]maltose (2 mCi $mmol^{-1}$), [1-^{14}C]Rib (2.6 mCi $mmol^{-1}$), and [1-^{14}C]Glc-6-P (4.9 mCi $mmol^{-1}$) were purchased from ICN and DuPont NEN. Technical data provided by ICN and DuPont NEN indicated that radiochemical purity was at least 99%. Trypsin type I (bovine pancreas), trypsin inhibitor type 1-S (soybean), and thermolysin type X (*Bacillus thermoproteolyticus*) were purchased from Sigma.

RESULTS AND DISCUSSION

Survey of Sugars Respired by Intact Chloroplasts from *Chlamydomonas*, Spinach, and Maize

There are some indications that the free sugars may be able to pass through the chloroplast envelope. Feeding of tobacco leaves (McLachlan and Porter, 1959) and of *Chlorella* (Kandler and Gibbs, 1959) with [1-^{14}C]Glc yielded starch in which the glycosyl units were labeled primarily in position 1, suggesting that the Glc entered the stromal compartment, the site of starch synthesis, without conversion to triose-P. Investigations utilizing methods such as osmosis (Wang and

Nobel, 1971) and transport of isotopically labeled sugar (Schafer et al., 1977) led to the conclusion that pea and spinach chloroplasts contain a specific carrier for transporting aldopentoses and aldo- and ketohexoses across the inner membrane of the chloroplast.

We investigated first whether the *Chlamydomonas*, spinach, and maize chloroplasts released CO_2 from externally supplied hexoses, Glc, Fru, Man, and Gal, a disaccharide, maltose, and a pentose, Rib, in the absence of ATP (Table I). The higher plant plastids evolved CO_2 from Fru, Glc, and Rib but essentially none from Gal, Man, and maltose. Fru and Rib were the preferred substrates in the spinach and maize chloroplasts, respectively. In the algal chloroplast, there was activity with all sugars, but Glc was the primary substrate.

There was an appreciable increase in the rate of CO_2 release from all sugars supplied to the *Chlamydomonas* chloroplast in the presence of external ATP (Table I). The most noticeable increase was observed with Glc. In the higher plant chloroplasts, the effect of ATP was restricted to Glc, Fru, and Rib.

CO_2 release reveals conversion of the free sugar to Glc-6-P and subsequent metabolism through the oxidative pentose-P pathway. Fru-6-P and Man-6-P resulting from the hexokinase reaction is converted to Glc-6-P by phosphoglucose isomerase and phosphomannose isomerase, respectively. Presumably, utilization of Gal involves a specific galactokinase that produces Gal-1-P with further metabolism by UDP-Gal pyrophosphorylase, UDP-Glc-4-epimerase, UDP-Glc pyrophosphorylase, and phosphoglucose mutase, resulting in the formation of Glc-6-P. The fact that C-1 of Rib is liberated as CO_2 possibly indicates action by a pentokinase and the formation of Glc-6-P from Rib-5-P processed through the oxidative pentose-P cycle.

There is no unequivocal explanation for the metabolic fate of maltose. Both the uptake of maltose (Herold et al., 1981) and its formation from hydrolytic starch breakdown (Heldt et al., 1977; Peavey et al., 1977) have been demonstrated in isolated spinach chloroplasts. Levi and Preiss (1978) and Okita et al. (1979) found no evidence that maltase is present in significant amounts in chloroplasts. On the other hand, there is a report of low levels of maltase phosphorylase activities in pea chloroplasts (Levi and Preiss, 1978). Our isotopic experiment indicates a very small amount of CO_2 released from maltose in the spinach chloroplast and a more substantial evolution in the algal chloroplast (Table I). The hazards of cellular fractionation of leaf tissue and isotopic methodology make it difficult to conclude that an enzyme is totally absent from the chloroplast. Inasmuch as maltose is not found in the chloroplast except in trace amounts during starch degradation, we suggest that its metabolism is catalyzed by maltose phosphorylase and perhaps a low level of maltase. Maltose could be exported from the chloroplast by a translocator (Herold et al., 1981). In contrast, an appreciable activity of maltose metabolism is associated with the *Chlamydomonas* chloroplast (Table I).

Table I. *Dark respiration of sugars in chloroplasts of C. reinhardtii F-60, spinach, and maize*

Included in the reaction mixture described in "Materials and Methods" were 525 μM Glc, 500 μM Fru, 400 μM Gal, 440 μM Man, 500 μM maltose, and 380 μM Rib. The Chl contents were 21, 78, and 31 μg for *Chlamydomonas*, spinach, and maize, respectively. The reactions were carried out in the dark at 25°C and were terminated after 30 min with perchloric acid.

Sugar	CO_2 Evolved					
	Chlamydomonas		Spinach		Maize	
	−ATP	+ATP	−ATP	+ATP	−ATP	+ATP
	nmol mg^{-1} of Chl h^{-1}					
[U-^{14}C]Glc	13	321	11	22	7	8
[U-^{14}C]Fru	23	38	20	48	9	14
[1-^{14}C]Gal	30	36	4	4	6	6
[1-^{14}C]Man	7	19	1	1	2	2
[U-^{14}C]Maltose	6	25	1	1	2	2
[1-^{14}C]Rib	14	25	7	18	15	47

Dependence of CO_2 Release on Fru and Glc Concentration

Inasmuch as the spinach chloroplast has been reported to host only a fructokinase (Schnarrenberger, 1990) and all published studies of higher plant (Stitt et al., 1978; Ahluwalia et al., 1989) and algal (Chen and Gibbs, 1991) chloroplasts involving the methodology of $^{14}CO_2$ release have been limited to externally supplied Glc, we compared the effects of increasing Glc and Fru concentrations on CO_2 evolution in the spinach chloroplast. The evolution of CO_2 showed saturation characteristics with respect to sugar concentration.

Under our standard assay conditions, the release of CO_2 in the absence and presence of ATP increased until the Fru and Glc concentrations were approximately 300 to 400 μM (data not shown). Apparent K_m values calculated from double-reciprocal plots of the data were found to be 125 μM for Fru and 200 μM for Fru and ATP. K_m values for Glc with and without ATP were 200 and 250 μM, respectively. K_m values of 140 μM for Fru and of 150 μM for Glc were reported for hexokinase purified from spinach leaves (Schnarrenberger, 1990). Glc uptake by the *C. reinhardtii* chloroplast was found to have a K_m of 48 μM (Chen and Gibbs, 1991).

Effect of Concentration of Mg^{2+} and ATP

In the presence of 2 mM ATP, increasing the $MgCl_2$ concentration increased the rate of CO_2 release from both Glc and Fru (data not shown). Maximum activity was obtained when the $MgCl_2$ concentration was approximately equal to that of ATP, and further increase in $MgCl_2$ resulted in an inhibition of respiratory activity. Turner and Copeland (1981) observed a similar response of increasing $MgCl_2$ concentration on purified pea seed hexokinase.

Effect of pH

The activity of our spinach chloroplasts in the evolution of CO_2 from Glc, Fru, and Rib in a series of buffers is shown in Figure 1. Clearly, chloroplast respiration in the dark was strongly dependent on pH. Whereas Glc had a maximal activity at about pH 8.5, Fru and Rib metabolism peaked at pH 7.5.

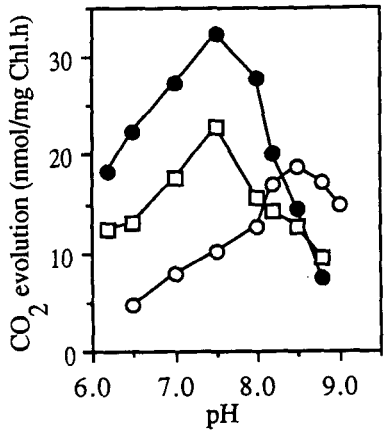

Figure 1. Effect of pH on release of $^{14}CO_2$ from [U-^{14}C]Glc, [U-^{14}C]Fru, and [1-^{14}C]Rib in spinach chloroplasts. Reaction mixtures were of the composition described in "Materials and Methods" except that 50 mM Mes-KOH (pH 6.2–6.5), 50 mM Hepes-KOH (pH 7.0–8.0), and 50 mM Tris-HCl (pH 8.2–9.0) buffers were used. Glc, Fru, and Rib concentrations were 525, 500, and 380 μM, respectively. The concentration of ATP was 2 mM. For the hexose experiment, the Chl concentration was 87 μg mL^{-1}, and for the Rib experiment, the Chl concentration was 112 μg mL^{-1}. ●, Fru; ○, Glc; □, Rib.

The pH optima cannot be explained solely on the basis of the sugar kinase reactions. Fructokinase and hexokinase purified from pea seed show a broad pH optimum between 7 and 9 (Turner and Copeland, 1981). We found a similar response for the phosphorylation of Glc and Fru in lysed spinach chloroplast preparations.

These findings may be explained in different ways: (a) Transport of the free and phosphorylated sugars require diverse translocators (Fig. 2). Translocation occurs through the action of a family of enzymes, each with specificity for a single substrate, rather than a single enzyme with a wide substrate specificity. (b) The metabolic routes are not common for the keto- and aldo-sugars. Further investigations will be necessary to decide between these possibilities.

Effect of Proteases on CO_2 Evolution

Several studies have utilized the proteases thermolysin and trypsin as surface probes of chloroplasts (Stitt et al., 1978; Cline et al., 1981; Joyard et al., 1983). The results suggest strongly that the effects of thermolysin and perhaps of trypsin treatment of intact chloroplasts are restricted to the outer membrane (Cline et al., 1984). Because the proteolytic enzymes would not be expected to cross the chloroplast envelope, treatment of isolated whole chloroplasts with thermolysin or trypsin would remove hexokinase if it were on the cytosolic side of the outer membrane but not if it were within the membrane. Removal of hexokinase should result in a decrease in the rate of $^{14}CO_2$ evolution from ^{14}C-sugar. To examine the effects of proteolytic enzymes, we incubated *Chlamydomonas* and spinach chloroplasts in thermolysin and trypsin and monitored $^{14}CO_2$ release from ^{14}C-sugars and [^{14}C]Glc-6-P. To provide evidence that the effects of the proteases were due to their proteolytic activities, samples were also incubated in trypsin plus trypsin inhibitor and thermolysin plus EGTA. This chelating agent blocks thermolytic activity by removing the required calcium ion. Treatment with the two proteases did not cause extensive damage, because photosynthetic CO_2 assimilation, an indicator of intactness, was approximately 75% of the rate in untreated whole chloroplasts (data not shown).

Incubation of *Chlamydomonas* chloroplasts with trypsin, but not with trypsin in the presence of trypsin inhibitor resulted in a decrease of about 70% in the rate of CO_2 release from all of the sugars (Table II). In spinach, tryptic digestion also reduced CO_2 release from Glc by a similar amount but had little effect when Fru was the substrate. These results were not affected by externally supplied ATP. Release of CO_2 from Glc-6-P was unaffected by trypsin, indicating that the multistep pathway including Glc-6-P dehydrogenase, gluconate-6-P, and the electron transport chain (Singh et al., 1992) remained. With respect to our assay, trypsin eliminated selectively the kinase reactions.

Our experiments with thermolysin were limited to the spinach chloroplast, and the data were essentially equivalent to those obtained with trypsin (Table III).

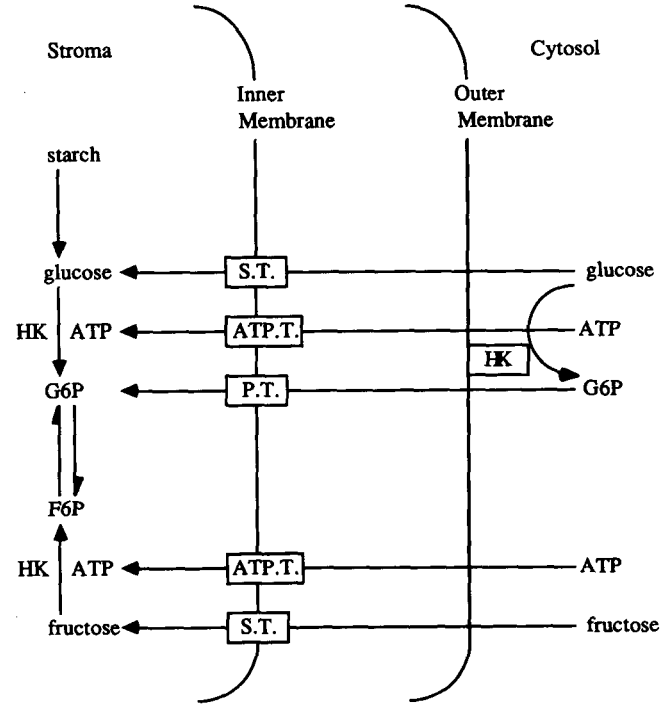

Figure 2. Scheme to show location of hexokinase in the stroma and on the outer membrane of the chloroplast. Glc and Fru are transported across the inner membrane by the sugar translocator (S.T.) and converted to Glc-6-P (G6P) and Fru-6-P (F6P), respectively, by stromal hexokinase (HK) with endogenous or with extrachloroplastic ATP. Glc is converted to G6P by the HK attached to the cytosolic side of the outer membrane with extrachloroplastic ATP. G6P and ATP are transported across the inner membrane by the phosphate translocator (P.T.) and the ATP translocator (ATP.T), respectively.

Table II. *Effect of trypsin on $^{14}CO_2$ release from ^{14}C-sugars and [1-^{14}C]Glc-6-P in intact chloroplasts of C. reinhardtii F-60 and Spinach*

Preparation of intact chloroplasts, treatment with trypsin, and $^{14}CO_2$ evolution measurements are described in "Materials and Methods." The concentration of ATP and Glc-6-P were 2 mM and 510 μM, respectively.

Sugar ± ATP	Treatment	CO_2 Evolution	
		Chlamydomonas	Spinach
		nmol mg^{-1} of Chl h^{-1}	
Glc	Control		8
Glc	Trypsin		3
Glc, ATP	Control	213	22
Glc, ATP	Trypsin	61	8
Glc, ATP	Trypsin and inhibitor	194	21
Fru	Control		23
Fru	Trypsin		20
Fru, ATP	Control	24	49
Fru, ATP	Trypsin	10	44
Man, ATP	Control	10	
Man, ATP	Trypsin	4	
Maltose, ATP	Control	16	
Maltose, ATP	Trypsin	5	
Glc-6-P	Control	107	68
Glc-6-P	Trypsin	108	58

Fractionation of the Spinach Chloroplast

The spinach chloroplast was subfractionated to localize hexokinase more definitively. Intact plastids were disrupted by osmotic shock and membranal fragments were sedimented at 50,000*g* for 15 min. The resulting supernatant (supernatant I) was the plastid stromal fraction, and the resuspended pellet, which was washed once with water and recentrifuged (supernatant II), was the plastid membranes (thylakoid and envelope). We used Glc-6-P dehydrogenase as a stromal marker enzyme.

Our data recorded in Table IV are consistent with a hexokinase being localized in the stroma of the chloroplast. The glucokinase activity of this enzyme was approximately 10% of the fructokinase activity. Of the total glucokinase activity, about half sedimented with the plastid membranes. In contrast, about 10% of the total fructokinase and Glc-6-P dehydrogenase was found in the insoluble fraction.

Table III. *Effect of thermolysin on $^{14}CO_2$ release from [^{14}C]Glc and [^{14}C]Fru in intact spinach chloroplasts*

Preparation of intact chloroplasts, treatment with thermolysin and calcium chloride, and $^{14}CO_2$ evolution measurements are described in "Materials and Methods."

Sugar ± ATP	Treatment	CO_2 Evolution
		nmol mg^{-1} of Chl h^{-1}
Glc	Control	7
Glc	Thermolysin, $CaCl_2$	3
Glc, ATP	Control	20
Glc, ATP	Thermolysin, $CaCl_2$	14
Fru	Control	16
Fru	Thermolysin, $CaCl_2$	12
Fru, ATP	Control	45
Fru, ATP	Thermolysin, $CaCl_2$	42

Table IV. *Glucokinase, fructokinase, and Glc-6-P dehydrogenase activities in subfractions of the spinach chloroplast*

Intact chloroplasts (440 μg of Chl ml^{-1}) were lysed in water and then centrifuged at 50,000*g* for 15 min, resulting in supernatant I and a pellet. The pellet was stirred in water and centrifuged at 50,000*g* for 15 min, yielding supernatant II and a pellet. Supernatants I and II and the pellet were analyzed for glucokinase, fructokinase, and Glc-6-P dehydrogenase activities. The data are the average of three experiments.

Fraction	Glucokinase	Fructokinase	Glc-6-P Dehydrogenase
	μmol mg^{-1} of Chl h^{-1}		
Supernatant I	1.1	8.1	16.2
Supernatant II	0	0.05	0.6
Pellet	1.9	1.2	1.2

Effect of Proteases on Glucokinase and Fructokinase Activities

There was a close correlation between the results recorded in Tables II through IV, indicating that glucokinase activity, in contrast to fructokinase activity, was localized primarily outside of the chloroplast. To provide additional evidence to support this compartmentation of the two activities, we incubated *Chlamydomonas* and spinach chloroplasts in thermolysin and trypsin and compared the effects on the activities of the two kinase reactions with Glc-6-P dehydrogenase and gluconate-6-P dehydrogenases, two dehydrogenases known to be present in the stroma and sensitive to proteolytic digestion. The sensitivity of the kinases to tryptic and thermolysitic digestion was established in parallel experiments carried out with lysed chloroplast preparations. As carried out previously, samples were also incubated in trypsin plus trypsin inhibitor and thermolysin plus EGTA.

About 85 to 90% of the total glucokinase activity associated with both chloroplasts (Tables V and VI) was destroyed by trypsin under conditions in which the activities of Glc-6-P dehydrogenase and gluconate-6-P dehydrogenase (data not shown) within the chloroplast remained largely unaffected. Approximately half of the fructokinase activity hosted by the intact spinach chloroplast remained (Table VI).

Chlamydomonas hexokinase (glucose as substrate), but not Glc-6-P dehydrogenase, was insensitive to thermolysin in both the intact and lysed chloroplast preparations (Table V). In the intact spinach chloroplast, of the total activities approximately 35% of the glucokinase and 70% of the fructokinase were recovered after thermolysin treatment (Table VI). Incubation with thermolysin up to 60 min did not affect the results (data not shown).

On the basis of published reports (Joyard et al., 1983; Cline et al., 1984) and our results, thermolysin treatment of intact chloroplasts is less damaging than treatment with trypsin. We speculate that thermolysitic activity was restricted to the

Table V. *Effect of trypsin and thermolysin on hexokinase and Glc-6-P dehydrogenase activities of intact and lysed chloroplasts from C. reinhardtii F-60*

Preparation of intact and lysed chloroplasts, treatment with trypsin and thermolysin, and assay of hexokinase (Glc was substrate) and Glc-6-P dehydrogenase are described in "Materials and Methods." The trypsin and thermolysin treatments were carried out with separate chloroplast preparations.

Treatments	Hexokinase		Glc-6-P Dehydrogenase	
	Intact	Lysed	Intact	Lysed
	μmol mg⁻¹ of Chl h⁻¹			
Initial activity	3.8	3.4	14.7	12.4
Trypsin	0.3	0	13.4	10.6
Trypsin and inhibitor	3.7	2.7	13.1	10.9
Initial activity	6.8	6.6	16.7	20.6
Thermolysin, $CaCl_2$	5.3	4.1	17.0	1.9
Thermolysin, $CaCl_2$, EGTA	6.6	6.2	16.1	16.9

outer membrane of the envelope. Trypsin, on the other hand, may well have penetrated the outer envelope membrane and hydrolyzed inner envelope membranal polypeptides. Neither, however, appeared to affect stromal enzymes.

CONCLUSION

Figure 2 is a scheme to account for our results obtained with the spinach chloroplast. We assign a hexokinase to the plastid stroma to explain the enzymic profiles and the release of CO_2 from Glc, Fru, and possibly Rib in the absence of externally supplied ATP. Based on the rate of CO_2 release and enzymic measurements, fructokinase activity is considerably greater than glucokinase activity. Clearly, the source of ATP under our conditions for this kinase reaction is chloroplastic. Transport of the free sugars is mediated by a carrier localized in the inner membrane (Schafer et al., 1977).

Our results also indicate that not all of the sugar kinase reactions were within the spinach chloroplast. Our results do not preclude the possibility that this hexokinase became attached to the chloroplasts during isolation. We argue against this view for two reasons: (a) washing the intact spinach chloroplasts three times in reaction medium did not decrease total hexokinase activity associated with the organelle; (b) incubation of intact chloroplasts with Fru-6-P and Glc-6-P did not dissociate hexokinase from the outer membrane as documented for mitochondria (Miernyk and Dennis, 1983).

To account for the 10 to 15% and 30% residual glucokinase measured enzymically and isotopically, respectively, in the *Chlamydomonas* chloroplast after treatment with trypsin, we suggest that, like for the spinach chloroplast, two pools of hexokinase (principally a glucokinase) exist, one in the stroma and one bound to the cytosolic side of the envelope. Inasmuch as the specific activities of the kinase reactions and of the oxidative pentose-P pathway vary less than 2-fold in the two chloroplasts, the 15-fold difference in CO_2 release from Glc requires another explanation. The striking dependency of the algal chloroplast on exogenous ATP for CO_2 evolution from Glc, we believe, is significant. Although the product of the glucokinase reaction does not readily cross the chloroplast envelope, we nevertheless stress that Glc-6-P does penetrate the chloroplast at rates (Heldt and Rapley, 1970) in excess of that needed to support the highest values of CO_2 released as recorded in this study. Our results suggest the possibility that most but perhaps not all of the Glc in the presence of the large pool of externally supplied ATP is phosphorylated by the glucokinase associated with the outer envelope membrane and Glc-6-P is transferred into the stroma by the phosphate or perhaps a hexose-P translocator.

Added ATP has only a small effect on CO_2 release in the spinach chloroplast. It follows that phosphorylation in the higher plant chloroplast is localized primarily in the stroma and that the delivery of extrachloroplastic ATP by an ATP-translocator protein restricts these reactions in the darkened chloroplast (Heldt, 1969).

Finally, the metabolic function of the sugar kinase reactions in spinach can be considered on the basis of their compartmentation. Glc generated by hydrolytic starch breakdown and documented in spinach (Heldt et al., 1977; Peavey et al., 1977) and in *Chlamydomonas* (Levi and Gibbs, 1984) would be returned to chloroplast metabolism by the stromal hexokinase. The hydrolytic rate of starch breakdown in the spinach chloroplast has been estimated to be about 10 to 25 nmol mg^{-1} of Chl h^{-1}. Our results indicate that the stroma has sufficient glucokinase capacity provided ATP is available to phosphorylate this rate of Glc formation.

A stromal source of Fru is as yet unknown in higher plants. Therefore, the physiological significance of the apparent preference of Fru versus Glc by the stromal spinach enzyme is not yet understood. In all likelihood, Fru is derived from cytoplasmic reactions (Huber and Akazawa, 1986; Pollack and Cairns, 1991) and is possibly phosphorylated by both hexokinases to provide substrates for the darkened chloroplast (Chen and Gibbs, 1992). Unfortunately, the dearth of

Table VI. *Effect of trypsin and thermolysin on hexokinase and Glc-6-P dehydrogenase activities of intact and lysed chloroplasts from spinach*

Preparation of intact and lysed chloroplasts, treatment with trypsin and thermolysin, and assay of hexokinase with Glc and Fru as substrate and Glc-6-P dehydrogenase are described in "Materials and Methods."

Treatments	Hexokinase				Glc-6-P Dehydrogenase	
	Glc		Fru			
	Intact	Lysed	Intact	Lysed	Intact	Lysed
	μmol mg⁻¹ of Chl h⁻¹					
Initial activity	2.5	3.4	9.5	10.2	15.5	14.5
Trypsin	0.2	0.2	4.7	1.1	12.6	1.0
Trypsin and inhibitor	2.2	3.0	9.5	8.9	15.4	14.3
Initial activity	2.4	1.8	10.4	9.6	17.0	17.0
Thermolysin, $CaCl_2$	0.9	0.5	6.8	1.4	15.9	3.3
Thermolysin, $CaCl_2$, EGTA	1.8	1.7	9.0	6.1	16.2	14.4

information concerning the functions and regulation of carbohydrate metabolism in the darkened chloroplast prevents definitive conclusions regarding the roles of the sugar kinase reactions hosted by the plastid.

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LITERATURE CITED

Ahluwalia KJK, Willeford KO, Gibbs M (1989) Aerobic and anaerobic respiration in the intact spinach chloroplast. Plant Physiol **90:** 653–656

Arnon DI (1949) Copper enzymes in isolated chloroplasts. Polyphenol-oxidases in *Beta vulgaris*. Plant Physiol **24:** 1–14

Baldus B, Kelly GJ, Latzko E (1981) Hexokinase of spinach leaves. Phytochemistry **20:** 1811–1814

Chen C, Gibbs M (1991) Glucose respiration in the intact chloroplasts of *Chlamydomonas reinhardtii*. Plant Physiol **95:** 82–87

Chen C, Gibbs M (1992) Coupling of carbon dioxide fixation to the oxyhydrogen reaction in the isolated chloroplast of *Chlamydomonas reinhardtii*. Plant Physiol **100:** 1361–1365

Cline K, Andrews J, Mersey B, Newcomb EH, Keegstra K (1981) Separation and characterization of inner and outer envelope membranes of pea chloroplasts. Proc Natl Acad Sci USA **78:** 3595–3599

Cline K, Werner-Washburn M, Andrews J, Keegstra K (1984) Thermolysin is a suitable protease for probing the surface of intact pea chloroplasts. Plant Physiol **75:** 675–678

Copeland L, Harrison DD, Turner JF (1978) Fructokinase (fraction III) of pea seeds. Plant Physiol **62:** 291–294

Doehlert DC (1989) Separation and characterization of four hexose kinases from developing maize kernels. Plant Physiol **89:** 1042–1048

Heldt HW (1969) Adenine nucleotide translocation in spinach chloroplasts. FEBS Lett **5:** 11–14

Heldt HW, Chon CJ, Maronde D, Herold A, Stankovic ZV, Walker DA, Kaminer A, Kirk MR, Heber U (1977) Role of orthophosphate and other factors in the regulation of starch formation in leaves and isolated chloroplasts. Plant Physiol **59:** 1146–1155

Heldt HW, Rapley L (1970) Specific transport of inorganic phosphate, 3-phosphoglycerate and dihydroxyacetone phosphate, and of dicarboxylates across the inner membrane of spinach chloroplasts. FEBS Lett **10:** 143–148

Herold A, Leegood RC, McNeil PH, Robinson SP (1981) Accumulation of maltose during photosynthesis in protoplasts isolated from spinach leaves treated with mannose. Plant Physiol **67:** 85–88

Huber SC, Akazawa T (1986) A novel sucrose synthase pathway for sucrose degradation in cultured sycamore cells. Plant Physiol **81:** 1008–1013

Jenkins CLD, Russ VJ (1984) Large scale rapid preparation of functional mesophyll chloroplasts from *Zea mays* and other C_4 species. Plant Sci Lett **35:** 19–24

Joyard J, Billecocq A, Bartlet SG, Block MA, Chua N-H, Douce R (1983) Localization of polypeptides to the cytosolic side of the outer envelop membrane of spinach chloroplasts. J Biol Chem **258:** 10000–10006

Kandler O, Gibbs M (1959) Untersuchungen über den Einfluss der Photosynthase auf die Austauschvorgange innerhalb des Hexosemolekuls. Z Naturforsch **14b:** 8–13

Klein U, Chen C, Gibbs M, Platt-Aloia KA (1983) Cellular fractionation of *Chlamydomonas reinhardtii* with emphasis on the isolation of the chloroplast. Plant Physiol **72:** 488–491

Kow YW, Robinson JM, Gibbs M (1977) Influence of pH upon the Warburg effect in isolated spinach chloroplasts. Plant Physiol **60:** 492–495

Levi C, Gibbs M (1984) Starch degradation in synchronously grown *Chlamydomonas reinhardtii* and characterization of the amylase. Plant Physiol **74:** 459–463

Levi C, Preiss J (1978) Amylopectin degradation in pea chloroplast extracts. Plant Physiol **61:** 218–220

McLachlan GA, Porter HK (1959) Replacement of oxidation by light as the energy source for glucose metabolism in the tobacco leaf. Proc R Soc Lond **150:** 460–473

Miernyk JA, Dennis DT (1983) Mitochondrial, plastid, and cytosolic isozymes of hexokinase from developing endosperm of *Ricinus communis*. Arch Biochem Biophys **226:** 458–468

Okita TW, Greenberg E, Kuhn DN, Preiss J (1979) Subcellular localization of the starch degradative and biosynthetic enzymes of spinach leaves. Plant Physiol **64:** 187–192

Peavey DG, Steup M, Gibbs M (1977) Characterization of starch breakdown in intact spinach chloroplasts. Plant Physiol **60:** 305–308

Pollock CJ, Cairns AJ (1991) Fructan metabolism in grasses and cereals. Annu Rev Plant Physiol Plant Mol Biol **42:** 77–101

Saltman P (1953) Hexokinase in higher plants. J Biol Chem **200:** 145–154

Schafer G, Heber U, Heldt HW (1977) Glucose transport into spinach chloroplasts. Plant Physiol **60:** 286–289

Schnarrenberger C (1990) Characterization and compartmentation in green leaves of hexokinases with different specificities for glucose, fructose, and mannose and nucleoside triphosphates. Planta **181:** 249–255

Singh KK, Chen C, Gibbs M (1992) Characterization of an electron transport pathway associated with glucose and fructose respiration in the intact chloroplasts of *Chlamydomonas reinhardii* and spinach. Plant Physiol **100:** 327–333

Stitt M, Bulpin PV, ap Rees T (1978) Pathway of starch breakdown in photosynthetic tissues of *Pisum sativum*. Biochim Biophys Acta **544:** 200–214

Turner JF, Copeland L (1981) Hexokinase II of pea seeds. Plant Physiol **68:** 1123–1127

Wang CT, Nobel PS (1971) Permeability of pea chloroplasts to alcohol and aldoses as measured by reflection coefficients. Biochim Biophys Acta **241:** 200–212

Willeford KO, Gibbs M (1989) Localization of the enzymes involved in the photoevolution of H_2 from acetate in *Chlamydomonas reinhardii*. Plant Physiol **90:** 788–791