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Thiol-Dependent Regulation of Glycerate Metabolism in Leaf Extracts[1]

THE ROLE OF GLYCERATE KINASE IN C_4 PLANTS

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LESZEK A. KLECZKOWSKI[2] AND DOUGLAS D. RANDALL*
Department of Biochemistry, University of Missouri, Columbia, Missouri 65211

ABSTRACT

We have recently reported that the activity of maize leaf glycerate kinase [EC 2.7.1.31] is regulated *in vivo* by the light/dark transition, possibly involving the ferredoxin/thioredoxin mechanism, and that the stimulating effect of light can be mimicked *in vitro* by incubation of crude leaf extract with reducing compounds (LA Kleczkowski, DD Randall 1985 Plant Physiol 79: 274–277). In the present study it was found that the time course of thiol activation of the enzyme was substantially dependent on the presence of some low molecular weight inhibitor(s) of activation found both in leaf extracts and mesophyll chloroplasts. Activity of glycerate kinase from maize as well as wheat leaves increased upon greening of etiolated plants and was correlated with the development of photosynthetic apparatus in these species. The maize enzyme was strongly activated by thiols at all stages of development from etiolated to green seedlings. Thiol activation of glycerate kinase was observed for a number of C_4 plants, notably of the nicotinamide adenine dinucleotide phosphate-malic enzyme type, with the strongest effect found for the enzyme from leaf extracts of maize and sorghum (10- and 8-fold activation, respectively). Among the C_3 species tested, only the enzyme from soybean leaves was affected under the same conditions (1.6-fold activation). This finding was reflected by an apparent lack of cross-reactivity between the enzyme from maize leaves and antibodies raised against purified spinach leaf glycerate kinase. We suggest that, in addition to its role as a final step of photorespiration in leaves, glycerate kinase from C_4 species may serve as a part of the facilitative diffusion system for the intercellular transport of 3-phosphoglycerate. Simultaneous operation of both the passive and the facilitative diffusion mechanisms of 3-phosphoglycerate transport in C_4 plants is postulated.

The operation of C_4 pathway of photosynthesis involves complex metabolic interactions, requiring transport of several compounds between the mesophyll and bundle sheath cells. The continuous transport of newly fixed C from the mesophyll to bundle sheath tissue is accomplished by either the malate/pyruvate or aspartate/alanine shuttle, depending on the C_4 species studied (9, 13, 14, 16). In some C_4 plants which have low PSII activity in bundle sheath cells (*e.g.* maize [6, 8]) and consequently have reduced capacity for $NADP^+$ photoreduction, an additional cooperation between both types of cells is required to sustain efficient regeneration of RuBP[3], the ultimate CO_2 acceptor in C_4 photosynthesis. While half of the NADPH needed for operation of the Calvin cycle in maize can be produced by the NADP-malic enzyme (decarboxylating), the other half has to be imported from mesophyll chloroplasts which contain a functional NADPH-producing system (8, 9, 14). This is accomplished by the 3-PGA/DHAP shuttle which provides reducing power required for photosynthesis in bundle sheath cells (13, 17).

Another flux of metabolites, also unique for C_4 metabolism, is the transport of the photorespiratory product, glycerate, from the bundle sheath to mesophyll cells. Despite the apparent lack of photorespiratory CO_2 evolution, leaves of C_4 species contain a full set of enzymes responsible for metabolism of phosphoglycolate formed by RuBP oxygenase activity (34). All but one of these enzymes are localized either exclusively or preferentially in the bundle sheath tissue (3, 34) and the only exception is GK, confined solely to mesophyll chloroplasts (36, 37). Studies with intact mesophyll chloroplasts demonstrated that glycerate could be easily taken up by these organelles and reduced to the triose-P level (37). Recent evidence from our own laboratory has indicated that the first step of glycerate metabolism in mesophyll chloroplasts of maize, formation of 3-PGA by GK, is light activated and some evidence for the involvement of the ferredoxin/thioredoxin mechanism in this process has been presented (21). The stimulating effect of light could be mimicked *in vitro* by incubation of the crude maize GK with reducing compounds (21).

In the present study we provide further evidence for thiol activation of GK from maize leaves and survey several C_3 and C_4 plants for the thiol effect on GK activity. Possible roles of GK in C_4 metabolism are discussed. A preliminary report describing some of the results obtained in the present study has been published (20).

MATERIALS AND METHODS

Plant Material. Maize (*Zea mays*) seeds were obtained from local seed supplier. Seedlings were grown as previously described (21).

Seedlings of tall fescue (*Festuca arundinacea* Schreb.), *Panicum boliviense* (*hylacecium*), pea (*Pisum sativum*), rye (*Secale cereale*), soybean (*Glycine max*), wheat (*Triticum aestivum*), *Panicum spathellosum* (*schenckii*), *Atriplex spongiosa*, *Brachiaria plantaginea*, crab grass (*Digitaria sanguinalis*), *Portulaca oleracea*, and *Sorghum bicolor* were grown in a greenhouse. Seedlings of green leaf lettuce (*Lactuca sativa*), *Andropogon* spp.,

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[2] Permanent address: Warsaw Agricultural University, Department of Plant Physiology, 02-528 Warsaw, Poland.

[3] Abbreviations: RuBP, ribulose-1,5-bisphosphate; DHAP, dihydroxyacetone phosphate; GK, glycerate kinase; 2-ME, 2-mercaptoethanol; PEP, phosphoenolpyruvate; PGA, phosphoglycerate.

bermuda grass (*Cynodon dactylon*), giant foxtail (*Sertaria faberi*), millet (*Panicum miliaceum*), sugarcane (*Saccharum officinarum*), and switch grass (*Panicum virgatum*) were grown in the field. Spinach (*Spinacia oleracea*) leaves were purchased in a local grocery store.

Reagents. Most biochemicals were from Sigma or PL-Biochemicals, Inc. All reagents were commercial preparations of the highest grade available. Phosphoglycerate phosphokinase (yeast) and glyceraldehyde phosphate dehydrogenase (rabbit muscle) were from Sigma.

Developmental Studies. Maize and wheat seeds were planted in flats of commercial Promix in a growth chamber and kept in the dark at 25°C. The 8-d-old etiolated seedlings were then exposed to constant fluorescent light (2×10^4 ergs/cm^2·s) for up to 50 h (25°C). At different stages of greening, leaf samples were collected and frozen with liquid N_2.

Isolation of Glycerate Kinase. Crude leaf GK from maize and other plants was isolated in non-reducing conditions in a grinding buffer containing 40 mM Tricine, pH 7.8, 2 mM $MgCl_2$ and 1 mM EDTA. For the developmental studies, prior to the actual extraction, samples of 0.5 to 1.0 g of maize and wheat leaves were frozen with liquid N_2 and then extracted in 2 to 3 ml of the grinding medium using mortar and pestle (0–4°C). For other studies, samples of leaves (7–15 g) were cut into 4 to 6 mm-wide slices and homogenized for 15 s in a Waring Blendor using 50 ml of the grinding medium (0–4°C). All homogenates were squeezed through 4 layers of cheesecloth and 1 layer of Miracloth and centrifuged at 10,000*g* for 10 min.

Mesophyll chloroplasts of maize were isolated as previously described (21).

Immunological Studies. Antibodies against GK were obtained by injecting a New Zealand white male rabbit with two doses of the homogeneous spinach enzyme, purified as previously described (22). First dose of the antigen contained 200 μg of spinach GK mixed with Freund's complete adjuvant (Cappel Laboratories) in a 1:1 ratio. Injections were done subcutaneously along the spine of the rabbit. Second dose of the antigen (200 μg), mixed (1:1) with Freund's incomplete adjuvant (Cappel Laboratories) was injected in 2 weeks after the first immunization. Blood was collected in 10 weeks following second injection. The IgG fraction, containing antibodies against GK, was obtained by chromatography of crude serum on DEAE-Affi-Gel Blue (Bio-Rad) column.

Details of immunoprecipitation studies are described in the legend to Figure 5. Slab gel electrophoresis was done according to Laemmli (23). The Western blotting technique was performed according to a slightly modified procedure of Towbin *et al.* (35), as described by Kleczkowski (18). For detection of the specific antigen-antibody complexes on nitrocellulose, the alkaline phosphatase-labeled goat anti-rabbit IgG (Sigma) was used as a second antibody.

Assay of Glycerate Kinase. Conditions of the assay of GK were as previously described (21). Assay (1 ml) contained 100 mM Tricine, pH 7.8, 5 mM ATP, 5 mM DL-glycerate, 10 mM $MgCl_2$, 0.2 mM NADH, various aliquots of GK and 5 units each of phosphoglycerate phosphokinase and glyceraldehyde phosphate dehydrogenase. Control assays containing all the components of the reaction but glycerate were run to correct for nonspecific oxidation of NADH. One unit of GK activity was expressed as 1 μmol NADH oxidized per min at 25°C.

Analytical Methods. Determination of protein and Chl was done according to Bradford (4) and Arnon (1) procedures, respectively.

RESULTS AND DISCUSSION

Thiol Activation of Maize Glycerate Kinase. Activity of maize GK was very susceptible to a treatment with reducing compounds. Incubation with 100 mM 2-ME or 20 mM DTT resulted in 6- to 7-fold activation of the enzyme isolated in non-reducing conditions (Fig. 1). DTE was found as effective as DTT in activation of maize GK, while incubation with 20 mM concentration of either reduced glutathione or cysteine had no effect on activity of the enzyme (data not shown). The time course of activation was strongly dependent on the amount of crude enzyme incubated with thiols: the more concentrated the leaf extract the longer the time period required to obtain highest activity (Fig. 1). These data suggested the presence of some endogenous inhibitor(s) of thiol activation of GK. To evaluate this possibility, different concentrations of the crude as well as crude-desalted enzyme were incubated for 16 min with 100 mM 2-ME, and then assayed for GK activity. The amount of proteins incubated with 100 mM 2-ME was plotted against the observed activation of GK (Fig. 2A). These plots showed that the rate of thiol activation of GK was strongly inhibited by increasing amounts of the extract. The extent of inhibition was much smaller for the crude-desalted extracts when compared to the crude preparations. Reciprocal plots for both crude and crude-desalted extracts were constructed to determine the maximal activation of GK after 16 min incubation with 100 mM 2-ME (Fig. 2B). In either case, inhibition of the thiol activation of GK was shown to be a linear function of endogenous concentration of the inhibitor(s), with the "apparent K_i" values of about 50 and 500 μg protein/ml extract for crude and crude-desalted preparations, respectively. The A_{max} value did not change upon desalting (Fig. 2B) and generally corresponded to the level of activation of GK after prolonged (2–6 h) incubation of the crude enzyme with 100 mM 2-ME. For different leaf preparations of maize, the range of GK activity for the minus thiol and plus thiol treatments was 7 to 12 and 50 to 90 nmol/min·mg protein, respectively.

The presence of some inhibitor(s) of thiol activation of GK was found for both leaf extracts (Fig. 2, A and B) and isolated mesophyll chloroplasts (Fig. 3) suggesting a chloroplast localization for the inhibitor(s).

At present it is unclear whether the observed inhibition of thiol activation of GK may have any significance with respect to the *in vivo* light regulation of the enzyme. Possibility of an artifact intimately associated with the isolation procedures of both leaf and chloroplast GK can not be ruled out. The inhibiting agent(s) might be any compound either directly preventing reduction of

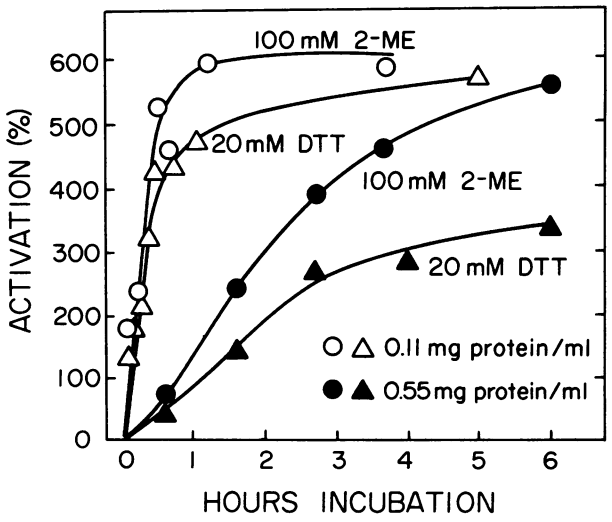

FIG. 1. Time course of thiol activation of maize leaf glycerate kinase. Leaves of 14-d-old maize seedlings were used. Crude extract was diluted to a given concentration of protein with the grinding medium and incubated with reducing compounds at 25°C. The basic activity of GK, assayed without thiols, was 8 nmol/min·mg protein.

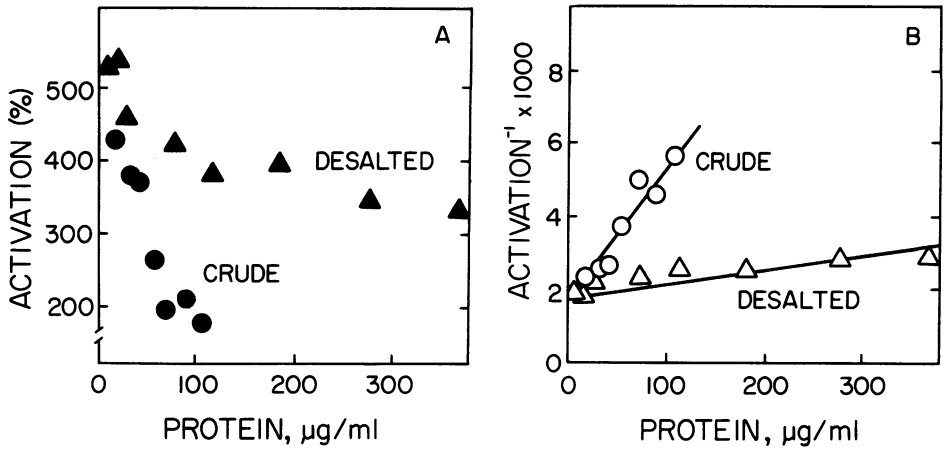

FIG. 2. Presence of some endogenous inhibitor(s) of thiol activation of glycerate kinase in maize leaf extracts. Leaves of 12-d-old seedlings were used. A, Various aliquots of crude or crude-desalted (by Sephadex G-25) leaf extract were incubated with 100 mM 2-ME for 16 min (1.0 ml total volume, 25°C) and then aliquots of 0.1 ml were assayed for GK activity. The basic activity, assayed without thiols, was 11 nmol/min·mg protein. B, Dixon plots of the data obtained in A.

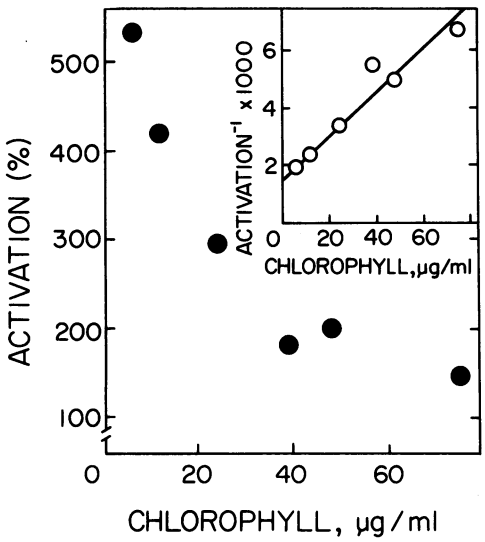

FIG. 3. Presence of some endogenous inhibitor(s) of thiol activation of glycerate kinase in maize mesophyll chloroplasts. Mesophyll chloroplasts were isolated as described in Kleczkowski and Randall (21) and stored at −20°C. For the thiol activation experiment, the fraction was thawed, centrifuged and then various aliquots of crude stromal extract were incubated with 100 mM 2-ME for 16 min (1.0 ml total volume, 25°C). Aliquots of 0.1 ml were assayed for GK activity. The basic activity, assayed without thiols, was 3.6 nmol/min·ml stromal extract. Concentration of Chl was determined before centrifugation. Inset presents Dixon plot of the data obtained in this experiment.

the disulfide bond(s) on the enzyme (e.g. quinones) or inducing a conformational change in GK structure and making disulfide bonds less available for reduction. In the latter case, it is interesting to note that ATP acts synergistically with the DTT-reduced thioredoxins in activation of partially purified maize GK (21). This suggests that the Fd-thioredoxin system of chloroplasts might function jointly with some other effector metabolites in regulation of maize GK *in vivo*.

Thiol Regulation of Plant Glycerate Kinase. Since in our previous study (21) we had found no thiol effect on the activity of spinach leaf GK (and no light activation as well), it was of interest to survey several plants for the thiol activation of GK and to establish how general this phenomenon was. Table I presents such a survey and compares activities and thiol activation of GK from C_3 and C_4 plants. Aliquots of crude leaf homogenates were desalted before assays to minimize possible involvement of the inhibitor(s) of thiol activation, as shown for maize extracts (Figs. 1 and 2). Besides maize GK, a marked stimulation by 2-ME was observed for the enzyme from leaves of sorghum (8-fold), sugarcane (4-fold) and *Andropogon* (3-fold). Some thiol activation was also found for GK from leaves of switch grass (1.7-fold) and soybean (1.6-fold). With the exception of soybean, all of these species are C_4 plants. On the other hand, 2-ME was ineffective in modulating the activity of GK from seven other C_4 and six C_3 plants as well as the only C_3-C_4 intermediate species tested, *Panicum spathellosum*. Rates of the activated GK in maize and sorghum, when expressed on whole leaf protein basis, constituted about 50% or more of the activity found for the enzyme from C_3 plants. Leaf preparations of green lettuce did not show any activity of GK, regardless of whether the enzyme was assayed without or with a high 2-ME concentration. It is unknown whether this finding reflects a lack of GK in lettuce or is a result of unfavorable conditions of extraction and/or assay of the enzyme from this particular species.

We have previously demonstrated (21) that preincubation of the partially purified maize GK with the DTT-reduced spinach thioredoxins *m* and *f* increases activity of the enzyme in an analogous fashion to the effect of thiols in crude leaf extracts. This was consistent with the observed light activation of maize GK from both intact leaves and mesophyll chloroplasts (21) and suggested involvement of the endogenous maize Fd-thioredoxin system *in vivo*. Activity of all the enzymes known to be regulated by the Fd-thioredoxin mechanism can usually be modulated *in vitro* by incubation of leaf extracts with DTT or other reductants (5). The strong thiol activation found for GK from maize and a number of other C_4 plants (Table I) suggests that light regulation of GK might be a common characteristic of these species. On the other hand, not all C_4 species exhibited regulation of GK by thiols and not all C_3 plants lacked this ability, as demonstrated for soybean. These apparent differences within C_4 and C_3 groups of plants warrant a careful and detailed further study, since several factors, such as light/dark conditions prior to extraction, pH of the grinding buffer, and availability of divalent cations (5), could decrease or eliminate susceptibility of GK to the thiol treatment. We did not attempt to optimize the conditions of either extraction, thiol treatment and/or assay of GK for each of the species studied.

Development of Glycerate Kinase upon Greening. Both maize and wheat plants, when grown in total darkness for 8 d and then transferred to the continuous light regime, showed an increase in GK activity upon greening (Fig. 4A). After 50 h in the light, activities of GK and Chl content in both plants had almost reached the values observed for leaves of control plants grown under normal light/dark conditions. The thiol-stimulated activity of maize GK, which probably represents the total amount of GK upon conditions of incubation and assay (21), increased about 50%, while the basic activity, determined without thiols,

Table I. *Activities and Thiol Activation of Glycerate Kinase from Leaves of C_3 and C_4 Species*

Before assays, the extracts were centrifuged and desalted by a passage through Sephadex G-25 column. Incubation with 100 mM 2-ME was performed directly in the assay mixture (minus glycerate) for 16 min.

Plant	Glycerate Kinase Activity		Activation
	−2-ME	+2-ME	
	units/mg protein·10^{-3}		*-fold*
C_3:			
Festuca arundinacea Schreb.	157.6	152.6	1.0
Glycine max	62.3	98.8	1.6
Lactuca sativa	0	0	
Panicum boliviense (*hylaecium*)	15.6	15.1	1.0
Pisum sativum	206.5	204.6	1.0
Secale cereale	190.0	186.4	1.0
Spinacia oleracea	246.2	248.5	1.0
Triticum aestivum	185.0	190.0	1.0
C_3–C_4:			
Panicum spathellosum (*schenckii*)	42.6	42.0	1.0
C_4:			
Andropogon spp.	21.1	66.4	3.1
Atriplex spongiosa	72.3	77.7	1.1
Brachiaria plantaginea	17.9	18.3	1.0
Cynodon dactylon	64.1	63.7	1.0
Digitaria sanguinalis	22.6	23.1	1.0
Panicum miliaceum	23.5	23.1	1.0
Panicum virgatum	7.3	12.5	1.7
Portulaca oleracea	7.2	8.0	1.1
Saccharum officinarum	14.3	59.7	4.2
Setaria faberi	54.5	53.0	1.0
Sorghum bicolor	10.5	85.3	8.1
Zea mays	9.0	87.3	9.7

rose 4-fold when compared to etiolated tissue. In contrast to maize GK, activity of the enzyme from wheat seedlings was not affected by the thiol treatment at any stage of development, and increased about 2.3-fold upon commencement of the light period (50 h). For either wheat and maize seedlings, the increase in GK activity upon greening was preceded by a 10 to 25 h lag phase, which corresponded to the lag in Chl synthesis (Fig. 4B).

The ratio of activities of maize GK for the +thiol/−thiol treatments decreased upon greening from 8:1 for etiolated seedlings to about 4:1 for plants at the end of illumination period (Fig. 4A). This decrease could result from the *in vivo* activation of the basic (−thiol) GK activity by light. Although the light-stimulated activity of maize GK is very unstable *in vitro* (unless low concentration of DTT is provided in the extraction medium [21]), it seems possible that the long-term irradiation, as utilized in the present study, may have had some stabilizing effect on the potential activity of the enzyme. The considerable increase in activities of either wheat and maize GKs and presence of a lag phase in their development suggest that GK is both physically and functionally associated with the photosynthetic processes in both C_3 and C_4 plants.

Immunological Characterization of Plant Glycerate Kinase. Immunoprecipitation of GK activity was performed to assess whether the previously reported (21) dissimilarities in properties of maize and spinach GK (M_r values, susceptibility to thiols, light activation) could be explained by differences in molecular structure of both proteins. We observed no precipitation of activity of maize GK by antibodies raised against the purified spinach enzyme (Fig. 5). Maize GK was not precipitated by the antibodies regardless of whether the enzyme was isolated in non-reducing or highly reducing (100 mM 2-ME) conditions. On the other hand, low amounts of serum were very effective in immunoprecipitation of GK from spinach as well as fescue leaf extracts (Fig. 5).

Lack of immunoprecipitation does not necessarily reflect lack of binding of specific antibody to the determinant site of an antigen. Therefore, we studied the binding affinity of the anti-spinach GK IgG using Western blot analysis following transfer of the SDS-PAGE resolved proteins onto nitrocellulose paper (Fig. 6). This study confirmed the lack of specific immunoreaction between maize GK and the antibodies. On the other hand, GKs from crude leaf extracts of wheat (Fig. 6) and spinach (data not shown) could easily be detected on the immunoblot, and their position on nitrocellulose corresponded to the M_r value of 40,000 which was analogous to the value previously obtained for purified spinach GK (19). The same M_r values found for GK from both etiolated and green seedlings of wheat indicate that the enzyme does not undergo any change in mol wt upon greening. Presence of a single band for GK from crude extracts of wheat and spinach is consistent with occurrence of only one isozyme of GK in leaves (32, 36, 37).

Possible Roles of Glycerate Kinase in C_4 Metabolism. Distinct characteristics of GK from C_3 and C_4 plants ([21] and present study) may reflect different roles of the enzyme in C_3 and C_4 metabolism. While GK from C_3 plants serves as a linkage between the oxidative and the reductive photosynthetic carbon cycles (22, 32). there does not appear to be a direct link between the two pathways in leaves of C_4 plants. GK is the only photorespiratory enzyme present exclusively in mesophyll cells of C_4 plants (36, 37) and, consequently, 3-PGA that originates from the glycolate pathway forms a separate pool from the 3-PGA synthesized by RuBP carboxylase/oxygenase activity. At least half of the latter pool may be reduced in bundle sheath cells (6, 14) and the remainder exported to mesophyll chloroplasts, which are the main source of reducing power in some C_4 plants (9, 14).

It is generally thought that the intercellular transport of 3-PGA

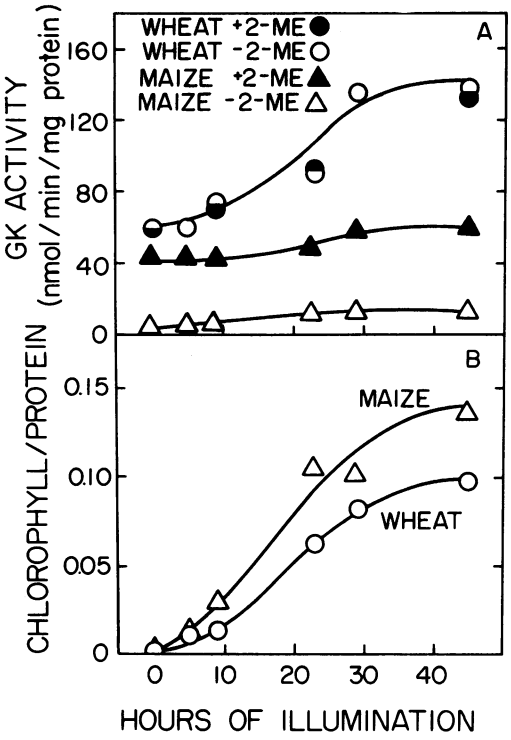

FIG. 4. Development of glycerate kinase activity (A) and Chl content (B) upon greening of etiolated maize and wheat seedlings. Before assays of GK, extracts were desalted on small Sephadex G-25 column. Incubation with 100 mM 2-ME was performed directly in the assay mixture (minus glycerate) for 16 min. The Chl/protein ratio was calculated as mg Chl/ml per mg protein/ml. For other experimental details, see "Materials and Methods."

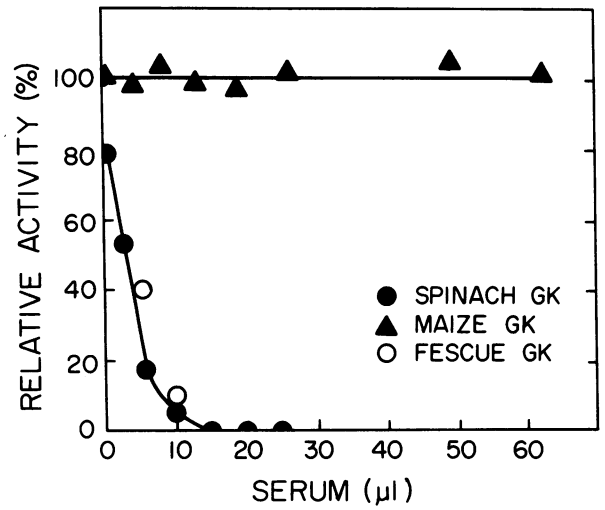

FIG. 5. Immunotitration of leaf glycerate kinase by antibodies against the spinach enzyme. Equal amounts of activity (3.1 nmol/min) of either spinach, maize or fescue crude leaf GK, isolated in nonreducing conditions, were mixed with the indicated volume of crude rabbit antiserum (total volume of each mixture was 0.2 ml) and incubated at 25°C for 40 min. Following incubation, mixtures were centrifuged at 10,000*g* for 5 min and the resulting supernatants used for assay of GK.

is accomplished by a build-up of this metabolite in the bundle sheath cells, followed by a passive diffusion along the developed gradient to the mesophyll tissue (14). It has been estimated that gradients of the order of 10 mM are required to allow rapid intercellular translocation of metabolites in C_4 plants (14). Re-

FIG. 6. Specific immunodetection of glycerate kinase from wheat and maize leaves following SDS-PAGE. Crude leaf extracts were loaded onto 12.5% polyacrylamide gel and electrophorized in SDS buffer. Following electrophoresis, resolved proteins were transferred onto nitrocellulose paper, treated with the rabbit anti-spinach GK IgG (1:100 dilution) and stained as described in "Materials and Methods." Lane A, etiolated wheat; lane B, etiolated maize; lane C, greening (29 h) maize; lane D, greening (29 h) wheat.

cent evidence by Leegood (24) tentatively suggests that the magnitude of the intercellular 3-PGA gradient in maize leaves is in fact adequate for the proposed simple diffusion mechanism. Also, rates of 3-PGA uptake into the mesophyll chloroplasts, when measured at optimal conditions, are sufficient to account for the net photosynthesis in C_4 plants (7, 17, 30, 37). On the other hand, the uptake of 3-PGA can be severely inhibited by a rather low concentration of Pi added to the incubation medium for chloroplasts (*e.g.* 0.5 mM Pi causing 40% inhibition at 0.71 mM 3-PGA; 2 mM Pi causing 25% inhibition at 9 mM 3-PGA [30]) which has been discussed as the evidence for existence of a common carrier for both metabolites (7, 11, 17, 30). However, assuming that the concentrations of Pi *in vivo* are in the range of 5 to 10 mM (31), which might be an underestimate (12), competition between 3-PGA and Pi for the same transporter may result in a lower efficiency of 3-PGA transport and decreased rates of its subsequent reduction in the stroma of mesophyll chloroplasts.

It has previously been suggested by Randall *et al.* (28) that the transport of 3-PGA could be accomplished by facilitative rather than passive diffusion. In the proposed scheme, 3-PGA would be hydrolyzed by 3-PGA phosphatase in the mesophyll cytosol and then glycerate and Pi taken up by the mesophyll chloroplasts and converted to 3-PGA by GK activity. Depletion of 3-PGA caused by the phosphatase would be a driving force for the flow

of this metabolite from the bundle sheath cells to the mesophyll cytosol. An interesting feature of this proposal is that glycerate used as a substrate by GK would not be derived from the glycolate pathway but from 3-PGA formed by RuBP carboxylase/oxygenase activity. Efficiency of the proposed scheme would require that the activities of both 3-PGA phosphatase and GK as well as the rates of transport of glycerate into the mesophyll chloroplasts stroma be sufficient to account for the massive flow of 3-PGA that is believed to occur between the bundle sheath and mesophyll cells. In this respect, while activity of 3-PGA phosphatase (100–600 μmol/h·mg Chl for various C_4 species [28]) seems sufficient for the proposed facilitative diffusion mechanism, it is not so for the activity of GK and probably for the rates of glycerate uptake as well. Rates of the thiol-activated GK in crude maize leaf extracts are in the range of 27 to 30 μmol/h·mg Chl (21), which is only about 20% of the rates found for the spinach enzyme (150–155 μmol/h·mg Chl [19, 32]). In spinach, the uptake of glycerate into the stroma (rate of about 40 μmol/h·mg Chl [29]) seems to be the limiting step of glycerate metabolism in chloroplasts. Rates of glycerate uptake into the mesophyll chloroplasts in C_4 plants are unknown, and the only information in this respect is the study by Usuda and Edwards (37) on the glycerate-dependent light-driven O_2 evolution from mesophyll chloroplasts of *Panicum capillare*. In their study, rates of about 20 μmol O_2 evolved/h·mg Chl were found for the glycerate-Pi cotransport, compared with rates of 80 μmol 3-PGA reduced/h·mg Chl for the 3-PGA-dependent O_2 evolution. These data clearly indicated that reduction of glycerate was not a limiting step in glycerate metabolism in the stroma. The magnitude of the glycerate-dependent O_2 evolution from mesophyll chloroplasts as well as the *in vitro* activity of the thiol stimulated GK are too low to regard the facilitative diffusion model as the sole mechanism of 3-PGA flow between the two types of cells.

In the light of the evidence presented above, we feel it is reasonable to propose that the flow of 3-PGA from the bundle sheath to mesophyll cells occurs by both passive and facilitative diffusion rather than by a single mechanism. Possible involvement of both mechanisms in the intercellular transport of 3-PGA may be discussed as a consequence of: (a) high activities of 3-PGA phosphatase in the mesophyll cytosol (28); (b) competition between 3-PGA and Pi for the common carrier in the inner membrane of mesophyll chloroplasts (7, 11, 17, 30); and (c) relatively high rates of light-activated GK in the stroma of these organelles (21). Because of the common carrier, the increased level of Pi formed by 3-PGA phosphatase (as well as PEP carboxylase) in the cytosol of mesophyll cells would decrease the amount of 3-PGA taken up into the stroma. On the other hand, glycerate formed from 3-PGA can also be transported through the chloroplast membranes (15, 29, 37), and does so in an equimolar ratio with Pi, providing balance of carbon for DHAP export to the cytosol (37). In such a scheme, glycerate would eventually be rephosphorylated by GK in the chloroplasts, regenerating 3-PGA hydrolyzed by the phosphatase in the cytosol. As previously proposed (21), the capability for light activation of GK from leaves of maize, and possibly some other C_4 species, could arise as an evolutionary response to the increased need for an effective flow of 3-PGA necessary to sustain the C_4-type of photosynthesis.

Coupled reactions of 3-PGA phosphatase and GK would increase the energy balance of C_4 photosynthesis by one ATP per each 3-PGA recovered in the mesophyll chloroplasts. Although expensive in terms of energy, participation of the facilitative diffusion system would provide a means for more effective and faster intercellular flow of 3-PGA, especially during photosynthetic induction. Another consequence of the proposed facilitative mechanism for 3-PGA transport is that the import of Pi into the stroma of mesophyll chloroplasts would be more effective: this seems important since Pi is needed for regeneration of PEP as well as photophosphorylation in these organelles (8, 13). In comparison to the simple diffusion model, simultaneous operation of both the passive and facilitative diffusion systems for 3-PGA transport would require smaller buildup of 3-PGA in the bundle sheath cells which might be important for regulation of some metabolic processes in this tissue. The operation of both passive and facilitative diffusion systems for 3-PGA transport in a C_4-metabolism plant is outlined in Figure 7.

Figure 7 does not include details of the metabolism of 3-PGA in mesophyll cells. It is clear, however, that following photosynthesis in $^{14}CO_2$-containing atmosphere the 3-PGA originating from RuBP carboxylase activity in bundle sheath cells and transported to mesophyll chloroplasts would retain its carboxyl label regardless of the mechanism of its intercellular transport. Therefore, activity of GK in C_4 plants may indirectly be involved in production of all the metabolites thought to originate from carboxyl-labeled 3-PGA in the mesophyll tissue (*e.g.* alanine [27], serine [26]).

It is unknown how much of glycerate metabolized by GK originates from 3-PGA hydrolyzed by the phosphatase in relation to the glycerate derived from glycolate. Although C_4 plants contain a complete set of photorespiratory enzymes, all methods to detect photorespiration by the whole leaf gas exchange experiments indicate that photorespiration is absent or very low in these species (34). On the other hand, low or no rates of apparent CO_2 evolution in the light do not necessarily reflect the steady state flow of metabolites throughout the glycolate pathway, since it is unknown how much of the photorespiratory CO_2 is refixed by RuBP carboxylase. The short time period required for maximal light activation of GK from maize leaves $t_{1/2}$ of about 1–2 min [21]) suggests the potential for rapid metabolism of glycerate during induction phase of C_4-photosynthesis. It seems unlikely, however, that the glycolate-derived glycerate can account even for a fraction of GK activity during first minutes of photosynthesis. In the view of generally low activities of photorespiratory enzymes in C_4 plants (3, 34), assuming passive flow of glycerate to the mesophyll tissue, one would expect that the buildup of glycerate in the bundle sheath cells would be much slower than the corresponding increase in levels of 3-PGA (approximately 20–30 min for development of sufficient gradient [24, 25]). In contrast to the glycolate-derived glycerate, at the early induction stage of photosynthesis in maize, there is a considerable buildup of 3-PGA in mesophyll cells, and it has been suggested that some part of this 3-PGA pool is formed *in situ* from PEP by the combined activities of enolase and P-glyceromutase (10). Since most, if not all, of these activities are confined to the cytosol (2, 33), the PEP-derived 3-PGA is a likely target for 3-PGA phosphatase, providing substrate for GK activity in the chloroplasts.

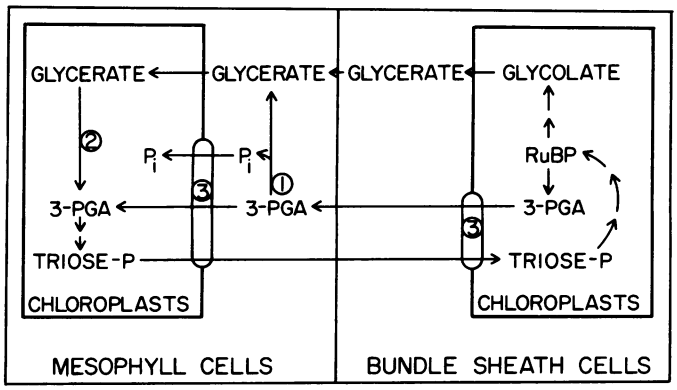

FIG. 7. Operation of both the simple diffusion and the facilitative diffusion systems for intercellular transport of 3-PGA in a C_4 plant—an outline. (1) 3-PGA phosphatase; (2) GK; (3) Pi translocator.

This and other evidence discussed above suggest that, at least during the induction stage of C_4-photosynthesis, glycerate utilized by GK originates most probably from 3-PGA rather than from the intermediate of the glycolate pathway.

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