

Light and Thiol Activation of Maize Leaf Glycerate Kinase¹

THE STIMULATING EFFECT OF REDUCED THIOREDOXINS AND ATP

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ABSTRACT

Glycerate kinase (EC 2.7.1.31) from maize (*Zea mays*) leaves was shown to be regulated by light/dark transition. The enzyme more than doubled in activity after either the leaves or isolated mesophyll chloroplasts were illuminated with white light for 10 minutes. Rate of inactivation in the dark was faster in leaves than in the isolated chloroplast fraction. The stimulating effect of light could be mimicked in crude preparations by addition of 10 or 50 millimolar dithiothreitol or 100 millimolar 2-mercaptoethanol. The thiol treatment resulted in 8- to 10-fold activation of glycerate kinase, with the highest rates in the range of 27 to 30 micromoles per mg chlorophyll per hour. Activation was not accompanied by any changes in the apparent M_r value of glycerate kinase as determined by gel filtration ($M_r = 47,000$). In contrast to maize glycerate kinase, the enzyme from spinach was not affected by either light or thiol exposure.

Partially purified maize glycerate kinase was activated up to 3-fold upon incubation with a mixture of spinach thioredoxins *m* and *f* and 5 millimolar dithiothreitol. The thioredoxin and dithiothreitol-treated glycerate kinase could be further stimulated by addition of 2.5 millimolar ATP. The results suggest that glycerate kinase from maize leaves is capable of photoactivation by the ferredoxin/thioredoxin system. The synergistic effect of ATP and thioredoxins in activation of the enzyme supports the earlier expressed view that the ferredoxin/thioredoxin system functions jointly with effector metabolites in light-mediated regulation during photosynthesis.

In plants showing C_4 type of metabolism (e.g. maize, sugarcane), the oxidative photosynthetic carbon pathway (photorespiration) is not directly linked to the Calvin cycle as in C_3 plants (e.g. spinach, wheat). In C_4 species, GK³—the terminal enzyme of photorespiration—is localized exclusively in the mesophyll cell chloroplasts (18), in contrast to all other photorespiratory enzymes which are compartmentalized either exclusively or preferentially in bundle sheath cells (4, 17). As a consequence of such a distribution, the phosphoglycolate metabolism up to the level of glycerate occurs in bundle sheath cells, while conversion of glycerate to 3-PGA is confined exclusively to mesophyll chloroplasts. The strict spatial separation of GK may indicate a

different role for this enzyme in C_4 plants when compared with the C_3 type of metabolism where a complete set of photorespiratory enzymes is found within a single cell (17), and where GK is considered solely as a linkage between the oxidative and the reductive carbon pathways of photosynthesis (10, 16). Other than its localization in mesophyll chloroplasts, little is known about GK from C_4 plants, and the enzyme has not been purified nor characterized from any of the C_4 species.

In this paper we present the data on light and thiol activation of GK from leaves of maize and provide evidence for the involvement of thioredoxin(s) in this process. Maize leaf GK is the first photorespiratory enzyme shown to be regulated by a light-mediated mechanism.

MATERIALS AND METHODS

Chemicals. DL-Glycerate and DL-DTT were obtained from Sigma. ATP and NADH were from PL-Biochemicals, Inc. All other reagents were commercial preparations of the highest grade available. Glyceraldehyde phosphate dehydrogenase (rabbit muscle), as well as phosphoglycerate phosphokinase (yeast) were from Sigma. The mixture of partially purified spinach leaf thioredoxins *m* and *f* (2 mg protein/ml stock) was generously provided by Dr. Bob B. Buchanan, University of California, Berkeley.

Plant Material. Maize (*Zea mays*) seeds were obtained from local seed supplier. Before sowing, the seeds were continuously washed with distilled H₂O for 3 to 5 h, and then stored at 0 to 4°C. Seedlings were grown in greenhouse in a commercial premix of 55% peat moss, 30% pumice, and 15% sand, and were watered daily with a nutrient solution containing 200 µg/ml nitrogen, 40 µg/ml phosphorus and 160 µg/ml potassium. For all experiments, the largest expanded leaves of the 9- to 13-d-old seedlings were used. Spinach leaves were purchased in a local grocery store.

Light Regulation of Leaf Glycerate Kinase. The 11-d-old maize seedlings were kept in the dark (25°C) for 5 h prior to the light/dark transition experiment. Plants were illuminated with white light provided by two 75-w Sylvania bulbs, and the light intensity measured at the level of top leaves was 550 µE·m⁻²·s⁻¹. At indicated times, about 1 g of leaves was quickly removed and frozen with liquid N₂. For enzyme extraction, the frozen leaf segments were rapidly homogenized using a chilled mortar with 10 ml of ice-cold medium containing 40 mM Tricine (pH 7.8), 2 mM MgCl₂, 1 mM EDTA, and 5 mM DTT. The homogenate was centrifuged at 10,000g for 2 min (0-4°C) and immediately assayed for GK activity. When DTT was omitted from the extraction medium, GK rapidly lost its original *in vivo* state of activation (data not shown), similarly to some other light-regulatable enzymes (19). Under conditions of the extraction (*i.e.* low temperature, short time period between extraction and assay), 5 mM DTT did not activate GK *in vitro*.

Light Regulation of Chloroplast Glycerate Kinase. Intact maize mesophyll chloroplasts were isolated as described by Anderson *et al.* (1) with the exception that DTT was omitted from

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³ Abbreviations: GK, glycerate kinase; 2-ME, 2-mercaptoethanol; PGA, phosphoglyceric acid.

isolation medium. The 20 g of 9-d-old maize seedlings (kept for 4 h in the dark before the experiment was started) were cut into 2- to 4-mm wide pieces in a small dish containing 200 ml of isolation medium. The isolation medium consisted of 30 mM Tes (pH 7.4), 0.33 M sorbitol, 1 mM EDTA, 1 mM MgCl₂, 1 mM MnCl₂, 0.5% BSA, and 2 g of Polyclar AT (insoluble acid-washed PVP). Leaf tissue was homogenized in a Braun AG blender (type MX 32) with razor-blade cutting blades at a maximum speed for 4 s. The brei was filtered through two layers of Miracloth, the filtrate centrifuged for 30 s at 300g, and the resulting supernatant centrifuged again for 10 min at 1,000g. The pellet was resuspended in 30 ml of isolation medium (minus Polyclar AT), equilibrated for 5 min, and centrifuged at 1,000g to yield mesophyll chloroplasts. After careful removal of the supernatant fraction, chloroplasts were resuspended in 8 ml of isolation medium (minus Polyclar AT). All steps leading to chloroplasts isolation were carried out at 0 to 4°C.

Intact spinach chloroplasts were isolated essentially as described by Reeves and Hall (15). Spinach leaves were darkened 4 h prior to the chloroplasts isolation.

For the light regulation experiments, after the exposure to light ($550 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ at 25°C), at indicated times the aliquots of either maize or spinach chloroplasts were broken open by dilution into ice-cold water (1:10 dilution). Extracts were centrifuged for 2 min at 10,000g and assayed for GK activity.

Isolation of Crude Leaf Glycerate Kinase. About 7 g of leaves of 9-d-old maize seedlings were cut onto 4- to 6-mm wide pieces and homogenized in a Waring Blender for 15 s (0–4°C) in 50 ml of grinding medium containing 40 mM Tricine (pH 7.8), 2 mM MgCl₂, and 1 mM EDTA. The homogenate was squeezed through 4 layers of cheesecloth and 1 layer of Miracloth, and centrifuged at 15,000g for 10 min. Resulting supernatant was subsequently referred to as 'crude GK'.

Partial Purification of Glycerate Kinase. Crude GK isolated from 10 g of 13-d-old maize seedlings was precipitated with the 30 to 75% saturated (NH₄)₂SO₄, and the pellet dissolved in a minimal volume of the grinding medium. The slurry was equilibrated for 10 min (0–4°C) and centrifuged to remove insoluble material. Supernatant fraction (3 ml) was chromatographed on a Sephadex G-75SF column (2.5 × 68 cm) which was equilibrated and eluted with 20 mM Mops (pH 7.0), and 2 mM MgCl₂. The 2-ml fractions were collected. The fraction showing highest GK activity was subsequently referred to as 'partially purified GK'.

Assay of Glycerate Kinase. GK activity was measured spectrophotometrically at 340 nm (25°C). The reaction mixture, unless otherwise indicated, contained, in 1 ml, 100 mM Tricine (pH 7.8), 10 mM MgCl₂, 5 mM ATP, 0.2 mM NADH, various aliquots of GK and 5 units each of phosphoglycerate phosphokinase and glyceraldehyde phosphate dehydrogenase. The reaction was initiated by addition of 5 mM DL-glycerate.

Analytical Methods. Chl and protein determination were done according to the procedures by Arnon (2) and Bradford (5), respectively.

RESULTS AND DISCUSSION

Thiol Activation of Glycerate Kinase. Maize leaf GK was markedly activated by addition of reducing compounds (Fig. 1). Incubation of crude leaf extracts with 10 or 50 mM DTT, or 100 mM 2-ME resulted in about 8- to 10-fold increase in activity of the enzyme. Maximal rates of maize GK when expressed on the chlorophyll basis were 27 to 30 $\mu\text{mol}/\text{mg Chl}\cdot\text{h}$. The half-time of activation was about 2 and 4 min for a treatment with 50 mM DTT and 100 mM 2-ME, respectively, and 12 to 15 min for 10 mM DTT. The time-course of thiol activation of GK was strongly dependent on the presence of some endogeneous inhibitor(s) of activation found both in crude leaf extracts and in the mesophyll chloroplast fraction (manuscript in preparation). In contrast to

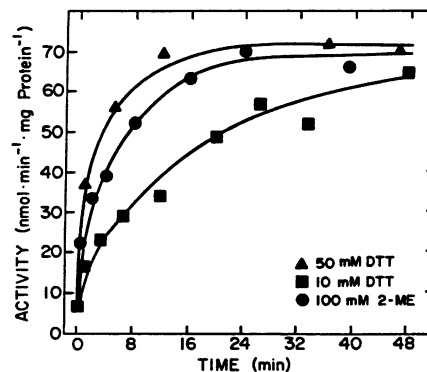


FIG. 1. Time-course of thiol-mediated activation of glycerate kinase from maize leaves. Crude leaf extract was desalted over a small Sephadex G-25 column and incubated with a given concentration of thiols at 25°C (0.21 mg protein/ml desalted extract). For other details see "Materials and Methods."

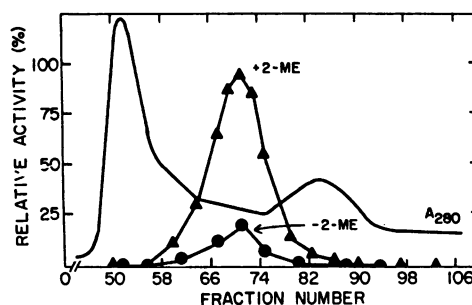


FIG. 2. Thiol activation of partially purified maize leaf glycerate kinase. (●, ▲); the activities of GK incubated with the content of the assay mixture (minus glycerate) for 16 min (25°C) in the absence and presence of 100 mM 2-ME, respectively. The value of 100% shown on the ordinate corresponds to the activity of 30 nmol/min·ml.

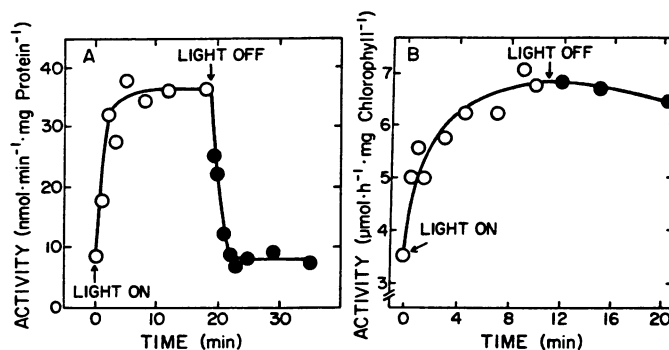


FIG. 3. Time-course of the light/dark regulation of glycerate kinase from leaves of maize (A) and from maize mesophyll chloroplasts (B). For experimental details see "Materials and Methods."

the maize enzyme, the activity of GK from spinach leaves (about $150 \mu\text{mol}/\text{mg Chl}\cdot\text{h}$) was not affected by incubation with reducing compounds (data not shown).

Maize GK which had been partially purified using Sephadex G-75SF column retained its capability for a thiol-mediated activation, showing about 5-fold increase in activity upon incubation with 100 mM 2-ME (Fig. 2). The estimated M_r value of the enzyme chromatographed over a calibrated Sephadex G-75SF column in nonreducing conditions was 47,000, while the M_r of 46,500 was found for the enzyme run over the same gel filtration column in presence of 100 mM 2-ME (data not shown). In the

latter case, GK was isolated in presence of 100 mM 2-ME. These data demonstrated that the interconversion between low activity (– thiol) and high activity (+ thiol) form of GK was not accompanied by any association/dissociation mechanism, and that maize GK was significantly larger than the corresponding proteins in C_3 plants (spinach GK – M_r 39,500 [9]; wheat and rye GKs – M_r 40,500 each [16]).

Light/Dark Regulation of Glycerate Kinase. Maize GK was rapidly activated upon illumination of leaves (Fig. 3A). The activity reached a plateau after about 5 to 8 min light exposure, with the highest rates being almost 4-fold higher than in the darkened leaves. Switching the plant from light to dark conditions resulted in an equally rapid loss of activity. A similar light-stimulation progress curve was obtained for GK activity using isolated mesophyll chloroplasts; however, the time-course of inactivation in the dark was much slower (Fig. 3B). This might indicate that the system responsible for dark inactivation of GK is disrupted in the isolated chloroplasts and therefore not as efficient as in intact leaf tissue. Another explanation might be that the inactivation phase requires an import or export of some effector(s) of GK from/to the cytoplasm and that biosynthesis and/or transport of such a compound would be stimulated by darkening of the leaves.

The light-treated maize GK from both leaves and intact chloroplasts could be further stimulated (100–200%) by incubation with 10 mM DTT or 100 mM 2-ME. This could indicate that the light-dependent mechanism was not sufficient to bring about the full activation of GK. On the other hand, conditions of the light exposure were not optimized. For instance, light intensity used in these experiments ($550 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) could be a limiting factor in the extent of light modulation of the enzyme. In C_4 plants, light intensity is normally considered a major limiting factor for photosynthesis under optimum temperature (17, 19). As has been shown by Usuda *et al.* (19), three light-activated enzymes from maize mesophyll chloroplasts—NADP-malate dehydrogenase, pyruvate, Pi dikinase, and fructose biphosphatase—required light intensities of 800 to $1,500 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ for maximal expression of their activities. At the $550 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ light intensity, activities of these enzymes were only about 55 to 60 % of the rates found at saturating light intensities.

We have failed to observe any light-modulable effect of GK from spinach chloroplast (data not shown).

At present it is unclear whether the light/dark regulation of GK is a distinct feature of the C_4 family of plants (*e.g.* maize), in contrast to species showing C_3 metabolism (*e.g.* spinach). Also, the significance of such a regulation is unclear. One might have argued that light stimulation of the metabolism of glycerate in the mesophyll chloroplasts is a means of protection of the photosynthetic apparatus in these organelles against photodestruction by an excess of ATP and NADPH produced during the light phase of photosynthesis. The role of photorespiration as a preventive mechanism against photoinhibition has previously been suggested for plants showing C_3 type of metabolism (8, 13). GK, together with PGA phosphatase, could also be involved in a facilitative diffusion system for 3-PGA transport as proposed by Randall *et al.* (14). Since C_4 plants supposedly evolved from C_3 species (11), it seems possible that the capability for light activation of GK arose in response to the increased need for an effective intercellular flow of 3-PGA necessary to sustain high rates of photosynthesis in C_4 species.

Effect of Reduced Thioredoxins and ATP. Both thiol and light activation of maize GK suggested that the ferredoxin/thioredoxin mechanism, mediating a transfer of reducing power from the photosynthetic electron transport system to some target proteins (6), could be involved *in vivo*. To test this hypothesis, the enzyme was partially purified by $(\text{NH}_4)_2\text{SO}_4$ precipitation followed by gel filtration, and then incubated with the DTT-reduced thioredox-

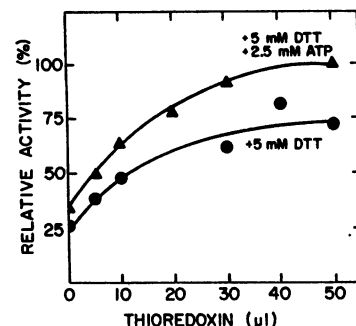


FIG. 4. Stimulation of maize leaf glycerate kinase by DTT-reduced thioredoxins *m* and *f* and ATP. GK was partially purified in nonreducing conditions as described in "Materials and Methods." The enzyme was incubated with 5 mM DTT \pm thioredoxins \pm 2.5 mM ATP at 25°C. After 16 min, each incubation mixture was assayed for GK activity. Final concentration of ATP in each assay was 5 mM. The value of 100% shown on the ordinate corresponds to the activity of 28 nmol/min·ml. See Table I for controls.

Table I. Effect of Reduced Thioredoxins *m* and *f* and ATP on Activity of Glycerate Kinase from Leaves of Maize

The data presented in this Table were obtained at the same time as the evidence shown in Figure 4. Experimental conditions were the same as in the legend to Figure 4.

Incubation Conditions	Relative Activity
	%
1. Enzyme	24
2. +Thioredoxins (40 μl)	24
3. +ATP (2.5 mM)	23
4. +DTT (5 mM)	25
5. +DTT (10 mM)	38
6. +Thioredoxins (40 μl) +ATP (2.5 mM)	24
7. +DTT (5 mM) +ATP (2.5 mM)	34
8. +Thioredoxins (40 μl) +DTT (5 mM)	72
9. +Thioredoxins (40 μl) +DTT (5 mM) +ATP (2.5 mM)	100 ^a
10. +2-ME (100 mM)	39
11. +2-ME (100 mM) +ATP (2.5 mM)	104

^a Estimated from Figure 4.

ins *f* and *m* from spinach leaves. The incubation resulted in about 3-fold increase in GK activity when compared to the control without reduced thioredoxins (Fig. 4). There was about 40% further increase in activity after 2.5 mM ATP had been included in the incubation mixture already containing the enzyme, thioredoxins, and 5 mM DTT. Correspondingly high rates could be obtained without reduced thioredoxins, when GK was incubated with 2.5 mM ATP and 100 mM 2-ME (Table I). On the other hand, 2-ME by itself was not as effective as with ATP, and increased the rates of the enzyme only about 60%. The control experiments indicate that the concentrations of either DTT, thioredoxins, and ATP did not by themselves change the activity of the enzyme (Table I). It should also be emphasized that an extension of the incubation time from 16 up to 60 min (data not shown) did not appreciably affect the rate observed with high (50 μl) content of reduced thioredoxins (Fig. 4), suggesting that in this time frame addition of ATP increases both the rate of activation as well as actual activity (V_{max}) of GK.

There has been growing evidence that the thioredoxin system

may function jointly with certain metabolite effectors in regulation of some photosynthetic enzymes *in vivo* (7). Recently, Ashton and Hatch (3) showed the inhibition of the thioredoxin-linked NADP-malate dehydrogenase activation by NADP, while Stitt and Heldt (cited in Buchanan [7]) reported the inhibition of thioredoxin-mediated activation of phosphoribulokinase by 6-Pgluconate. There has also been evidence that effectors can further increase the thioredoxin-stimulated activity, as reported for the effect of fructose-1,6-bisP and sedoheptulose-1,7-bisP on the activation of maize leaf fructose biphosphatase and sedoheptulose biphosphatase, respectively (12, 20). Stimulating effect of ATP observed for the thioredoxin-linked maize GK activation seems to be an additional example of the complex interactions between cell metabolites, enzymes, and the thioredoxin system. Since, in the presence of ATP, either 2-ME- or thioredoxin-catalyzed stimulation of GK yields similar rates of the enzyme (Table I), it would seem that ATP reacts with GK rather than with thioredoxin(s). Formation of the enzyme-ATP binary complex seems to facilitate reduction of GK by either thioredoxin(s) or high 2-ME content, which in turn results in some conformational change on the enzyme, leading to increased rates of catalysis. Without ATP in the incubation medium, the reduced thioredoxins were much more effective in activating the partially purified enzyme than 2-ME or DTT alone. The thiols, however, were very effective in activation of GK from crude rather than partially purified preparations. These data indicated that in crude extracts the enzyme was activated by some 2-ME- or DTT-reduced mediator rather than by 2-ME or DTT alone.

The *in vitro* activation of maize GK by spinach leaf thioredoxins strongly suggests that similar mechanism might be involved *in vivo*, with endogenous maize thioredoxin(s) mediating a transport of reducing power from thylakoids to the enzyme during light conditions. Both the thiol as well as light activation demonstrated for maize GK are consistent with the involvement of the ferredoxin/thioredoxin-like mechanism. On the other hand, ineffectiveness of the thiol treatment with respect to spinach GK is compatible with the observed lack of light modulation of this enzyme.

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