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Activation of the Potato Tuber ADP-glucose Pyrophosphorylase by Thioredoxin*

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The potato tuber (*Solanum tuberosum* L.) ADP-glucose pyrophosphorylase (ADP-GlcPPase) catalyzes the first committed step in starch biosynthesis. The main type of regulation of this enzyme is allosteric, and its activity is controlled by the ratio of activator, 3-phosphoglycerate to inhibitor, P_i. It was reported (Fu, Y., Ballicora, M. A., Leykam, J. F., and Preiss, J. (1998) *J. Biol. Chem.* 273, 25045–25052) that the enzyme was activated by reduction of the Cys^{12} disulfide linkage present in the catalytic subunits. In this study, both reduced thioredoxin *f* and *m* from spinach (*Spinacia oleracea*) leaves reduced and activated the enzyme at low concentrations (10 μM) of activator (3-phosphoglycerate). Fifty percent activation was at 4.5 and 8.7 μM for reduced thioredoxin *f* and *m*, respectively, and 2 orders of magnitude lower than for dithiothreitol. The activation was reversed by oxidized thioredoxin. Cys^{12} is conserved in the ADP-GlcPPases from plant leaves and other tissues except for the monocot endosperm enzymes. We postulate that in photosynthetic tissues, reduction could play a role in the fine regulation of the ADP-GlcPPase mediated by the ferredoxin-thioredoxin system. This is the first time that a covalent mechanism of regulation is postulated in the synthesis of starch.

ADP-glucose pyrophosphorylase (ADP-GlcPPase)[1] (ATP:α-D-glucose-1-phosphate adenylyltransferase, EC 2.7.7.27) catalyzes the first committed step toward the synthesis of starch in plants and bacterial glycogen (Reaction 1).

$$\alpha\text{-Glc-1-P} + \text{ATP} \xleftrightarrow{Mg^{2+}} \text{ADP-Glc} + PP_i$$

REACTION 1

This reaction is considered a prime regulatory and rate-limiting step (1–4). *In vitro*, since the reaction is reversible, the enzyme also catalyzes the pyrophosphorolysis of the ADP-Glc to produce ATP and Glc-1-P.

The potato (*Solanum tuberosum*) tuber enzyme, as all the ADP-GlcPPases from higher plants studied so far, is a heterotetramer comprised of two distinct subunits (5). Both of these genes were cloned and expressed in *Escherichia coli* to yield a recombinant enzyme comprised of two large (51 kDa) subunits and two small (50 kDa) subunits (L_2S_2) (3, 6). The amino acid sequence of the small subunits of higher plant ADP-GlcPPases is highly conserved, but the similarity among different large subunits is lower (7). The major function of the large subunit is to modulate the regulatory properties of the small subunit, which is primarily involved in catalysis (6, 8, 9). In every plant tissue studied so far, the major activator of the enzyme is 3-phosphoglycerate (3-PGA) and the major inhibitor is orthophosphate (P_i) (2, 10). In photosynthetic organisms, the ratio [3-PGA]/[P_i] regulates allosterically this reaction and therefore the synthesis of starch (10).

It was found that the activity of both the recombinant and the ADP-GlcPPase purified from potato tubers is stimulated by dithiothreitol (DTT) (6, 11). Recently, it was possible to characterize with the recombinant enzyme the mechanism of this stimulation (12). There are two distinct stages, activation and catalysis (Scheme I). The first one is a slow step that involves DTT and the substrates to convert the enzyme from a native (oxidized) to an activated reduced form. DTT is responsible for the reduction of the only disulfide bond present in the oxidized form, which is an intermolecular bridge between Cys^{12} of the two small subunits. The substrates are needed to attain the active conformation after the reduction. This process was tested in the absence of activators and inhibitors in the catalytic step. The ratio [3-PGA]/[P_i] is, however, still the main factor to regulate the activity of the enzyme. The reductive activation by DTT increased the activity up to 4-fold (12), whereas 3-PGA activated the enzyme 30-fold (8). The Cys^{12} is conserved in all small subunits from plant ADP-GlcPPases known so far with the exception of the ones from endosperm tissue. The role of the activation by DTT was not clear since it is not a physiological compound. However, the regulation of many enzymes in plants follows a similar scheme of activation, as depicted in Scheme I, where the physiological reductant is thioredoxin.

In this study, we tested both thioredoxin *m* and *f* from spinach (*Spinacia oleracea*) leaves. We observed that their reduced forms are able to activate the ADP-GlcPPase from potato tuber 4-fold when assayed at low concentrations of 3-PGA. We postulate that thioredoxin could play a role as a fine regulator of the ADP-GlcPPase from potato and from other plant tissues having the conserved Cys on the N-terminal of the small subunit.

MATERIALS AND METHODS

Reagents

[^{32}P]PP_i was purchased from NEN Life Science Products. [^{14}C]Glucose-1-P was from ICN Pharmaceuticals Inc. (Costa Mesa, CA). All other reagents were purchased at the highest quality available.

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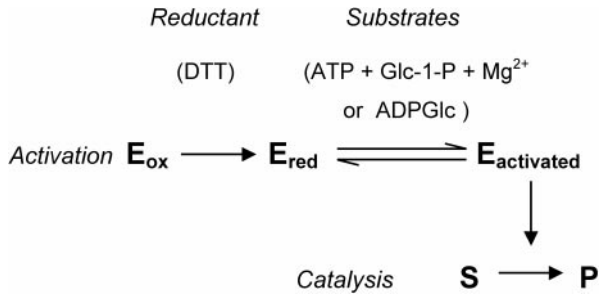
[1] The abbreviations used are: ADP-GlcPPase, ADP-glucose pyrophosphorylase; ADP-Glc, adenosine diphosphate glucose; Glc-1-P, glucose 1-phosphate; 3-PGA, 3-phosphoglycerate; DTT, dithiothreitol; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis.



SCHEME I.

DTT Concentration

DTT concentration was determined spectrophotometrically at 412 nm using 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent) as described previously (13).

Expression and Purification of the Potato Tuber ADP-GlcPPase

The recombinant ADP-GlcPPase from potato tuber was expressed in a strain of *E. coli* cell, AC70R1-504, devoid of endogenous ADP-GlcPPase activity. The enzyme was purified as indicated previously (6). It has been shown that the purified enzyme was in the oxidized form (12), and unless otherwise indicated, this enzyme form is referred to as the "oxidized" form.

Protein Assay

Protein concentration of the ADP-GlcPPase was determined by using bicinchoninic acid reagent (14) (Pierce) with BSA as the standard.

Reduction of the ADP-GlcPPase in Absence of Thioredoxins

The ADP-GlcPPase was reduced as follows: the purified enzyme (14 μg) was incubated in the presence of 50 mM Hepes (pH 8.0), 6 mM $MgCl_2$, 2 mM ADP-Glc, 0.5 mg/ml BSA, and 4 mM DTT at 37 °C in a total volume of 100 μl. After 30 min the enzyme was desalted using with Bio-spin 6™ columns (Bio-Rad) equilibrated with 50 mM Hepes (pH 8.0), 6 mM $MgCl_2$, 2 mM ADP-Glc and used immediately. It has been shown that in this condition the Cys^{12} is reduced (12).

Assay of ADP-GlcPPase

Pyrophosphorolysis Direction—Pyrophosphorolysis of ADP-Glc was determined by the formation of [^{32}P]ATP from $^{32}PP_i$. Unless otherwise indicated, the reaction mixture contained 20 μmol of glycine-glycine buffer (pH 8.0), 1.75 μmol of $MgCl_2$, 2.5 μmol of NaF, 0.5 μmol of ADP-Glc, 0.38 μmol of $^{32}PP_i$ (0.5 to 2.0 $\times$ 10^6 cpm/μmol), 50 μg of crystalline bovine serum albumin, and variable concentrations of 3-PGA in a final volume of 0.25 ml. The reaction was started by the addition of the enzyme and, after 2 min incubation at 37 °C, was terminated by the addition of 3 ml of cold 5% trichloroacetic acid. The [^{32}P]ATP formed was measured as described previously (15).

Synthesis Direction—Synthesis of ADP-Glc was followed by the formation of [^{14}C]ADP-Glc from [^{14}C]Glc-1-P. Unless otherwise indicated, the reaction mixtures contained, in 0.2 ml, 20 μmol of Hepes buffer (pH 8.0), 1.4 μmol of $MgCl_2$, 0.1 μmol of [^{14}C]Glc-1-P (1.0 $\times$ 10^6 cpm/μmol), 0.3 μmol of ATP, 0.3 unit of inorganic pyrophosphatase, and 40 μg of crystalline bovine serum albumin. 3-PGA was added at different concentrations as indicated. Assays were initiated by addition of the enzyme. Reaction mixtures were incubated for 5 min at 37 °C and terminated by heating in a boiling water bath for 1 min. [^{14}C]ADP-Glc was assayed as described previously (16).

SDS-PAGE Under Non-reducing Conditions and Immunoblot

SDS-PAGE was performed as described by Laemmli (17) on 4–15% Tris-HCl pre-cast gradient polyacrylamide gel (Bio-Rad) except that β-mercaptoethanol was not added to the protein samples. Molecular weight markers were Perfect™ protein markers (Novagen, Madison WI). After the electrophoresis, the gel was electroblotted onto a Protran™ nitrocellulose membrane (Schleicher & Schuell). This nitrocellulose membrane was treated with affinity purified anti-spinach leaf ADP-GlcPPase antibody (primary antibody), and the antigen-antibody complex was detected via treatment with alkaline phosphatase-linked goat anti-rabbit IgG. Color detection of the alkaline phosphatase was performed as described previously (18). Molecular weight markers were visualized including S-protein alkaline phosphatase conjugate (Novagen, Madison, WI) on the incubation with the secondary antibody.

Thioredoxin Purification

Recombinant thioredoxins *f* and *m* were purified as described in the literature (19) and kept frozen at −80 °C in small aliquots. The concentration was determined spectrophotometrically using an extinction coefficient at 278 nm of 16,830 and 20,500 M^{-1} cm^{-1} for thioredoxins *f* and *m,* respectively (19).

Reduction and Oxidation of Thioredoxins

Purified thioredoxins were incubated 5 min at 37 °C in the presence of 50 mM Hepes (pH 8.0), 0.1 mM EDTA, and 5 mM of either reduced or oxidized DTT. 50 μl of this reaction was desalted with Bio-spin 6™ columns (Bio-Rad) equilibrated in 50 mM Hepes (pH 8.0) and used immediately. An aliquot of the eluate was separated and diluted in a 50-μl quartz cuvette to measure the concentration of thioredoxin as mentioned above. This procedure efficiently removed the DTT from the samples. A control with reduced DTT but no thioredoxin showed no detectable amounts of DTT after the desalting step (<4 μM) using 5,5′-dithiobis(2-nitrobenzoic acid) as described above.

Kinetic Characterization

Kinetic data were plotted as velocity *versus* substrate or effector concentration. Kinetic constants were obtained by nonlinear least square fitting of the data with the Hill equation using the program Origin™ 5.0 (Microcal Software Inc., Northampton, MA). Kinetic constants were expressed as $A_{0.5}$, $S_{0.5}$, $I_{0.5}$, which correspond to the concentration of effector necessary to reach 50% of maximal activation, velocity, and inhibition, respectively. The Hill coefficient was represented as *n*.

RESULTS

Reduction of the ADP-GlcPPase by Plant Thioredoxins—The ADP-GlcPPase from potato tuber was sensitive to reduction by both of the reduced thioredoxins tested. It has been shown that the small subunit of the potato tuber ADP-GlcPPase migrates as a dimer in non-reducing SDS-PAGE when the intersubunit disulfide bridge Cys^{12}-Cys^{12} is present (oxidized form) and as monomer when it is cleaved (reduced form) (12). The large subunit, which has no disulfide bridge, runs in both conditions as a monomer (12). Therefore, a change of the redox state of the heterotetrameric enzyme can be detected clearly from a modification of the electrophoretic mobility of the small subunit. The antibody (anti-spinach leaf ADP-GlcPPase) used in this work to develop the immunoblot after the electrophoresis is more reactive against the small subunit than the large subunit from potato tuber ADP-GlcPPase. When the enzyme was incubated for 15 min in the presence of ADP-Glc, Mg^{2+}, and either thioredoxin *f* or *m*, changes in the electrophoretic mobility were observed (Fig. 1). The lower band, which corresponds to the monomers, was much stronger after the incubation, whereas the upper band that constitutes the dimer of the small subunit almost disappeared. When the enzyme was incubated with oxidized thioredoxin, no significant difference was observed with respect to the control. This indicates that the reduced form of both thioredoxins were responsible for the change of mobility of the ADP-GlcPPase, which is attributable to the reduction of the enzyme. Concurrently, these changes in the redox state of the ADP-GlcPPase correlated with the activity in the pyrophosphorolysis direction. When the enzyme was reduced by either thioredoxin, the activity increased more than 2-fold when assayed in absence of the allosteric activator 3-PGA (Fig. 1).

Affinity of the Effectors for the Reduced and Oxidized Forms of the ADP-GlcPPase—Since the reduced thioredoxins *f* and *m* can activate the enzyme in the pyrophosphorolysis direction, it was of interest to test the properties of the reduced and oxidized forms of the ADP-GlcPPase in the ADP-Glc synthesis direction, which is the physiological reaction. Once the enzyme was reduced in presence of thioredoxin *f*, the activity was assayed at different concentrations of 3-PGA and compared with the enzyme treated in absence of thioredoxin (control). It

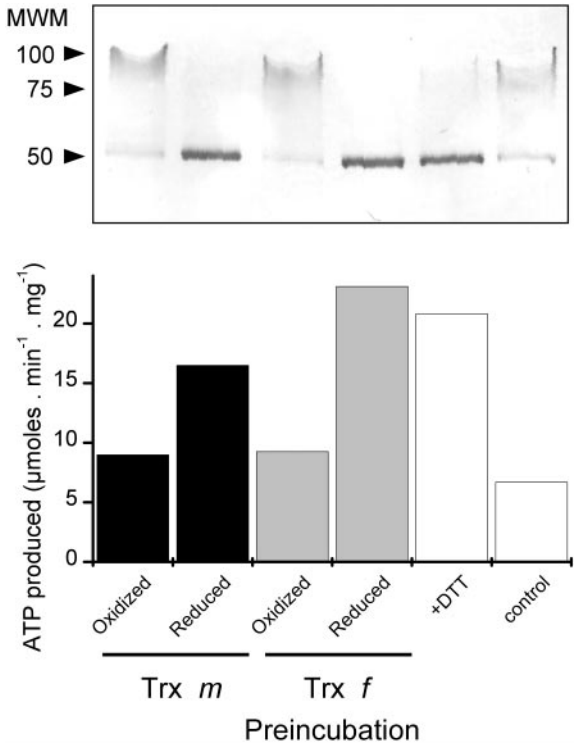


FIG. 1. **Reduction of the potato tuber ADP-GlcPPase by thioredoxins and activation of the pyrophosphorolysis.** In the control experiment, the ADP-GlcPPase was incubated 15 min at 37 °C in 60 μl of a solution containing 50 mM Hepes (8.0), 1.6 mM ADP-Glc, and 1 mM $MgCl_2$. The other tubes contained the same as the control and either 5 mM DTT or 28 μM thioredoxin (*Trx*) that had been previously reduced or oxidized as described under "Materials and Methods." After the incubation a 10-μl aliquot (0.96 μg) was withdrawn for assaying the activity in the pyrophosphorolysis direction in the absence of 3-PGA and DTT. Another 10-μl aliquot of the incubation mixture was mixed with 10 μl of 0.2 M iodoacetic acid and was subsequently run in an SDS-PAGE under non-reducing conditions. The immunoblot was performed as described under "Materials and Methods." Activity assays were performed in duplicate, and variations were less than 10% in all cases. *MWM* refers to molecular weight markers.

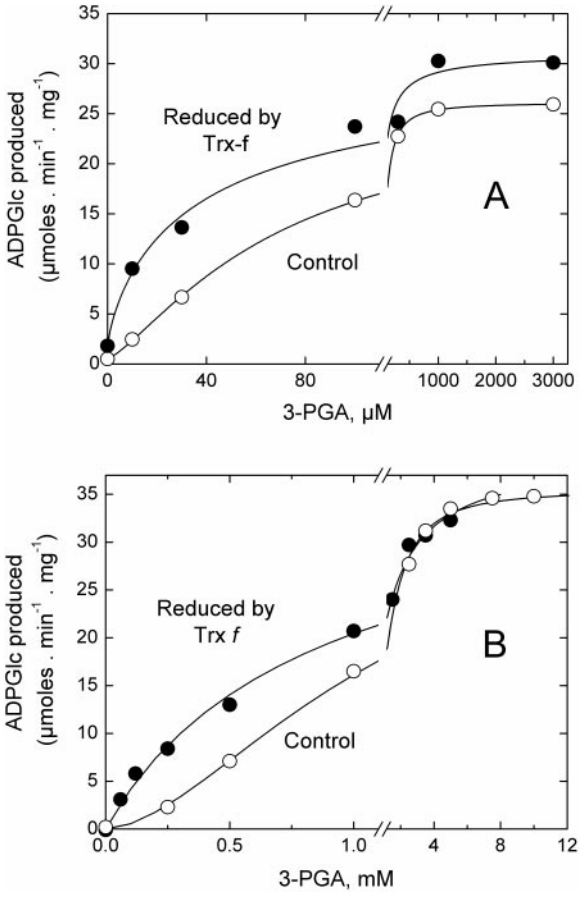


FIG. 2. **Activation by 3-PGA of the potato tuber ADP-GlcPPase reduced by thioredoxin *f*.** The enzyme (1.4 μg) was reduced in 100 μl of a solution containing 50 mM Hepes (pH 8.0), 6 mM $MgCl_2$, 2 mM ADP-Glc, and 12 μM of reduced thioredoxin *f* (*Trx f*) at 37 °C for 30 min. The control experiment followed the same procedure, but no thioredoxin was added. After the incubation, aliquots containing 0.047 μg of ADP-GlcPPase were assayed in the synthesis direction at different concentrations of 3-PGA as described under "Materials and Methods." *A,* there was no P_i in the assay mixture; *B,* the concentration of P_i was kept constant at 2 mM. Each point was determined in duplicate, and the average was plotted. Results for each point varied less than 10%.

was observed that the thioredoxin-reduced ADP-GlcPPase has a higher affinity for the activator (Fig. 2). The $A_{0.5}$ was 40 μM for the reduced and 85 μM for the control enzyme. Since the curve of the control enzyme was slightly more sigmoidal (n = 1.5) than the curve of the activated enzyme (n = 0.8), the activity at lower concentrations of activator was markedly higher for the thioredoxin-reduced ADP-GlcPPase. When the concentration of 3-PGA was 10 μM in the assay mixture, the activity of the thioredoxin-reduced enzyme was more than 4-fold higher than the control. However, both of the forms of the ADP-GlcPPase, reduced and oxidized, did not show big differences in the parameters of other effectors: the main inhibitor (P_i), substrate (Glc-1-P) and the cofactor (Mg^{2+}) (Table I). Only the substrate ATP showed a significant but rather small difference since the apparent affinity of the reduced form was 1.6-fold higher (Table I). The $S_{0.5}$ values were 56 and 90 μM for the reduced and oxidized forms, respectively. In presence of P_i, there is a shift on both 3-PGA activation curves, but the effect of the thioredoxin is maintained. At 0.2–0.3 mM 3-PGA the difference in activity between the reduced and the oxidized form is ~4-fold.

TABLE I
Kinetic parameters of the reduced and oxidized ADP-GlcPPase from potato tuber

The ADP-GlcPPase was used in its oxidized form as described under "Materials and Methods" or was first reduced in 50 mM Hepes (pH 8.0), 6 mM $MgCl_2$, 2 mM ADP-Glc, and 12 μM reduced thioredoxin *f* at 37 °C for 30 min. Aliquots were withdrawn, and the activity was determined in the synthesis direction. Concentration of the effector was varied, whereas the concentration of the others was kept constant. The concentration of the activator 3-PGA was 10 μM in all cases. The kinetic parameters were determined as described under "Materials and Methods." The deviation is the difference between duplicate experiments.

Effector	ADP-GlcPPase form	
	Oxidized	Reduced by thioredoxin *f*
	$I_{0.5}$ *(n), μM*	
P_i	120 ± 20 (0.9)	140 ± 25 (0.9)
	$S_{0.5}$ *(n), μM*	
ATP	90 ± 9 (1.1)	56 ± 3 (1.2)
Glc-1-P	32 ± 2 (1.7)	34 ± 2 (1.2)
Mg^{2+}	1830 ± 60 (4.5)	1640 ± 80 (7.0)

Kinetics of Reductive Activation—The time curve of the reductive activation was studied assaying the enzyme in the synthesis direction in presence of 10 μM 3-PGA. This concentration of activator maximizes the difference between both redox forms of the ADP-GlcPPase (Fig. 2). The enzyme incubated in the presence of 12 μM thioredoxin *f* increased the activity up to 4-fold reaching near-maximum after 30 min. The calculated $t_{0.5}$ of activation was 14 min. On the other hand, the control incubation did not modify the activity of the enzyme even after 60 min (Fig. 3), which further demonstrates that the

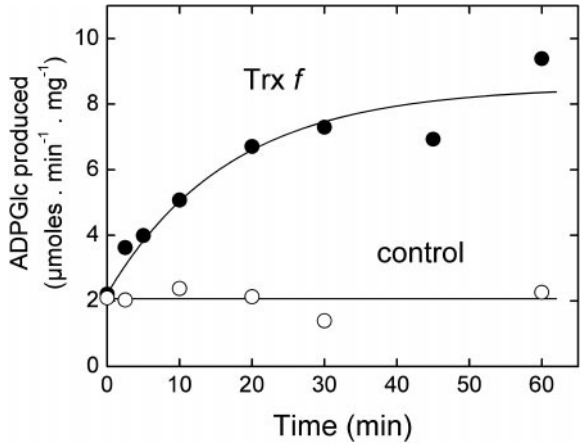


FIG. 3. **Kinetic of activation by reduced thioredoxin *f* of the potato tuber ADP-GlcPPase.** The enzyme (28 μg) was incubated in 200 μl of a solution containing 50 mM Hepes (pH 8.0), 6 mM $MgCl_2$, 2 mM ADP-Glc, and 12 μM of reduced thioredoxin *f* (*Trx f*) at 37 °C. At different times, a 5-μl aliquot was withdrawn and immediately assayed in the synthesis direction for 2 min in presence of 10 μM 3-PGA. The control experiment followed the same procedure, but no thioredoxin was added.

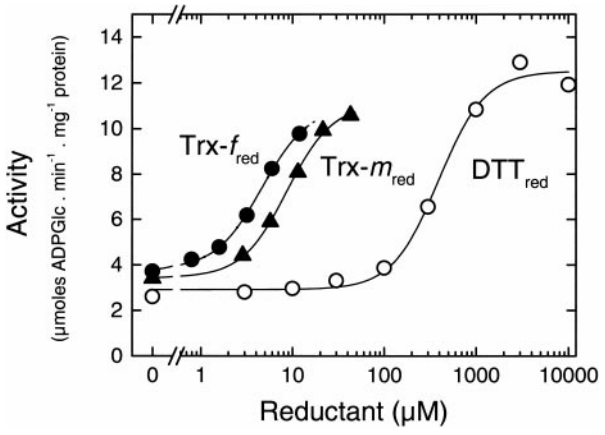


FIG. 4. **Activation of the potato tuber ADP-GlcPPase by different concentrations of reductants.** The enzyme (1.4 μg) was incubated at 37 °C in 50 μl of a solution containing 50 mM Hepes (pH 8.0), 6 mM $MgCl_2$, 2 mM ADP-Glc, and different concentrations of reductants. After 30 min, aliquots of 10 μl were withdrawn and assayed in presence of 10 μM 3-PGA in the synthesis direction as indicated under "Materials and Methods." Each point was determined in duplicate, and the average was plotted. *Trx*, thioredoxin; *red,* reduced.

presence of reduced thioredoxin is needed for the activation.

Activation Curve of Reductants—Both reduced thioredoxins *f* and *m* were capable of activating the ADP-GlcPPase from potato tuber. However, it has been observed that other reducing agents (monothiols and dithiols including DTT) have similar effects on the enzyme.[2] To test if the thioredoxins transfer the reducing power more efficiently, the reaction was performed at different concentrations. It was observed that both thioredoxins were needed in concentrations 2 orders of magnitude lower than DTT, which is the most powerful reducing agent tested on this enzyme. Thioredoxin *f* was twice as effective as *m*. The $A_{0.5}$ values were about 4.6, 8.7, and 400 μM for thioredoxin *f, m,* and DTT, respectively (Fig. 4).

Reversibility of the Reductive Activation—The reversibility of the process was examined incubating the activated (reduced) ADP-GlcPPase in the presence of oxidized thioredoxin. It was observed that oxidized thioredoxin *f* inactivates the enzyme. After 30 min of incubation, the activity was only 42% when compared with the control that was in absence of thioredoxin (Table II). This inactivation was completely reversible because after the addition of DTT the activity increased 4.2-fold (Table II), which is the ratio that is expected between the oxidized and reduced form (Figs. 3 and 4). It was observed that after adding DTT the activity increased 1.7-fold in the control (Table II) indicating that the enzyme was not completely reduced. Most likely, the control was partially oxidized by O_2 during the 30-min incubation.

TABLE II

Inactivation of the reduced ADP-GlcPPase by oxidized thioredoxin

The potato tuber ADP-GlcPPase (2.8 μg), previously reduced as indicated under "Materials and Methods," was incubated in presence of 20 mM Hepes (pH 8.0), 6 mM $MgCl_2$, 2 mM ADP-Glc, and 12 μM oxidized thioredoxin *f* at 37 °C. After 30 min, an aliquot (10 μl) was withdrawn to determine the activity in the synthesis direction in the presence of 10 μM 3-PGA (B). In the control experiment (A), the oxidized thioredoxin *f* was not included in the incubation mixture. After the aliquots were withdrawn, DTT was added to the incubation mixture to reach a concentration of 6 mM. The incubation proceeded for 15 min at 37 °C, and the activity was assayed as described above (C and D). All assays were performed in duplicate, and the average is shown in the table. The deviation is the difference between the duplicates.

Incubation	Activity
	$\mu mol \cdot min^{-1} \cdot mg^{-1}$
A, Control	5.6 ± 0.2
B, thioredoxin f (oxidized)	2.4 ± 0.1
C, A, then + DTT + 15 min	9.4 ± 0.2
D, B, then + DTT + 15 min	10.1 ± 0.4

DISCUSSION

It had been previously shown in our laboratory that the potato tuber ADP-GlcPPase could be activated by DTT and that the activation mechanism involved the slow reduction of the Cys^{12}-Cys^{12} intersubunit disulfide bridge (12). In this work, we show that the enzyme is also activated by chloroplast thioredoxins *f* and *m* from spinach leaves and that either of the two reduced plastidial thioredoxins can replace DTT in the cleavage (Fig. 1) of the disulfide bridge. The kinetic properties of the thioredoxin-reduced enzyme differ from those of the oxidized form, as shown by a change in the affinity toward the activator 3-PGA (Fig. 2). Measurement of behavior toward other effectors did not detect any other important differences between the oxidized and reduced forms when assayed in the presence of 10 μM 3-PGA (Table I). This indicates that the affinity for the substrates does not play a role on the reductive activation of the enzyme at low concentrations of activator. Moreover, the affinity for the substrates does not vary whether the enzyme is activated by 3-PGA, reduction, or both. When the enzyme was assayed at saturated concentrations of 3-PGA (2 mM) the K_m values for ATP and Glc-1-P were not significantly different between the reduced and oxidized forms and were similar to the values shown in Table I (data not shown). ADP-Glc was included in the activation mixture since it had been shown previously, when DTT was used as activator, that there was a further, conformational, conversion after the chemical, reductive, step if the substrate site is fully occupied (12). Filling of substrates sites can be achieved using ATP, Glc-1-P, and Mg^{2+}/Ca^{2+} or ADP-Glc.

Thioredoxin *f* proved to be to be more efficient than thioredoxin *m* in the activation process, but the difference was only 2-fold. Although other enzymes regulated by thioredoxin seem to be far more selective toward one or another of the two forms, *f* or *m* (20), in some cases it has been shown that the inclusion of enzyme effectors diminished the selectivity significantly (21). The $A_{0.5}$ for the activation of the potato tuber ADP-GlcP-

[2] M. A. Ballicora, J. B. Frueauf, Y. Fu, P. Schürmann, and J. Preiss, unpublished results.

Pase by spinach leaf thioredoxin *f* was 4.6 μM, higher than the $A_{0.5}$ for the most potent interactions between thioredoxin and its specific enzyme targets like fructose-1,6-bisphosphatase and thioredoxin *f* (0.9 μM) (22), but also much lower than nonspecific interactions between fructose-1,6-bisphosphatase and *E. coli* thioredoxin (33 μM). Recently, it was shown that the main factor in the specificity of thioredoxins *m*, *f,* and bacterial thioredoxins is manifested by electrostatic interactions (22). When the interactions are shielded or abolished by mutagenesis, the $A_{0.5}$ tends to reach values of ~10 μM in all cases. It is possible that the forms *m* and *f* interact in a similar fashion with the potato tuber ADP-GlcPPase because the surface of the enzyme is neutral in the region of contact, but the three-dimensional structure of the enzyme would be needed to confirm this assumption. We do not discard the possibility that other factors (pH, the presence of 3-PGA, P_i, etc.) can modify the conformation of the enzyme exposing charged amino acids and making the interaction more selective, but this hypothesis has not been tested.

It has been shown that the homology of the first 20 amino acids at the N terminus of the small subunits of the plant ADP-GlcPPases studied is very high, and the amino acids conserved include the Cys^{12} responsible for the reductive regulation of the potato tuber enzyme. The exceptions to the rule include the monocot endosperm ADP-GlcPPases (23). An interesting case is barley (*Hordeum vulgaris*) where there is a mechanism of splicing that produces two ADP-GlcPPases corresponding to different tissues. The two enzymes share the same sequence except for the first 17 amino acids at the N terminus (24); the leaf enzyme has a Cys on the N terminus of the small subunit but the endosperm enzyme does not.

Regarding the physiological relevance of the regulation by thioredoxin, it is worth noting that the same small subunit gene of the potato ADP-Glc PPase is expressed both in tuber and leaf (25), although the level of expression is higher in tuber. Both enzymes are located in plastids; the leaf form is in the chloroplast, and the tuber enzyme is in the amyloplast (26). The most important physiological difference between these two subcellular environments is that the chloroplast is autotrophic whereas the amyloplast is heterotrophic (27). In chloroplasts, the physiological interpretation of the regulation by thioredoxin *in vitro* is straightforward; the ferredoxin-thioredoxin system, which consists of ferredoxin, ferredoxin-thioredoxin reductase, and thioredoxin, is located in the chloroplast, and it has been thoroughly characterized (20). Upon illumination, photosynthetic electron transport reduces ferredoxin, which then reduces thioredoxin via the ferredoxin-thioredoxin reductase. Reduced thioredoxin regulates many enzymes by this light-mediated mechanism, and at night, the effect of thioredoxin, oxidized by O_2, is the opposite.

The results presented in this paper suggest that thioredoxin could reduce or oxidize the chloroplastic ADP-GlcPPase during the light/dark cycle, thus providing a fine- tuned regulation of starch synthesis on chloroplasts. This mechanism could be particularly important at low concentrations of 3-PGA, where the difference of activity between the reduced and oxidized forms is maximal. The [3-PGA]/[P_i] ratio would be the main factor in the regulation, but in the dark, when the concentration of 3-PGA decreases, the oxidized thioredoxin could lower even more the activity of the ADP-GlcPPase, thus avoiding a futile cycling of starch metabolism. Indeed, oxidative inactivation of the ADP-GlcPPase by oxidized thioredoxin *f* occurred *in vitro* (Table II). In absence of P_i it was observed that the optimal concentration of 3-PGA to observe the regulation by thioredoxin is ~10 μM. When the assay is performed in presence of 2 mM P_i, there is a shift on the 3-PGA activation curves, but the effect of regulation by thioredoxin in the lower part of the curve (0.2–0.6 mM) is conserved (Fig. 2*B*). This is closer to the physiological conditions in the plastids. In amyloplasts, it is not clear what concentrations are physiological for these two metabolites. In chloroplasts, the concentration of P_i is difficult to determine experimentally because of the large amounts stored in the vacuoles; however, it is believed that both 3-PGA and P_i concentrations are in the mM range (28). The light/dark change produces shifts of 1 pH unit in the chloroplast (20). Although the data reported here were obtained at pH 8.0, we observed that the reductive regulation by thioredoxin *f* also occurs at pH 7.0 and 7.5 (data not shown).

The potato ADP-GlcPPases from tuber and leaf are heterotetrameric, and although the small subunit is the same in both enzymes (25), the large subunits are different. It is the small subunit that contains the site subject to reductive regulation, but the modulation exerted in the leaf enzyme on the small subunit by the different large subunit might give to the leaf form different kinetic constants than those reported here for the tuber enzyme. It would be interesting to test the effect of the plastidial thioredoxins (*m* and *f*) on the enzyme from potato leaf. Earlier experiments by Kaiser and Basham (28) suggested that the ADP-GlcPPase in an extract of lysed spinach leaf chloroplasts could be activated by reduction. High levels of DTT (10–50 mM) increased the synthesis of ADP-Glc in these extracts 5.7-fold (28), but the interpretation of those experiments was not clear because conditions for the activation of the purified enzyme were not reported (10).

The relevance of the regulation by thioredoxin is not clear for an enzyme that is located in the amyloplast like the ADP-GlcPPase from potato tuber. Moreover, thus far it has not been investigated whether there is any thioredoxin in amyloplast or any other heterotrophic plastid. This is because biochemical studies on amyloplasts have been very few when compared with studies on chloroplasts, mainly because amyloplasts are very difficult to isolate intact. Despite the lack of experimental evidence, it is likely that a thioredoxin form is present in amyloplasts since thioredoxins are widely distributed and have been detected in many plant tissues and in different subcellular locations, including cytosol, mitochondria, chloroplasts, and nuclei (29). Indeed, plants display more diversity of thioredoxins than other organisms. For instance, in *Arabidopsis thaliana,* there are at least eight thioredoxin *h* genes, and four thioredoxin *m* and two thioredoxin *f* forms have been isolated (29). The other issue is what is the nature of the reducing power that would drive regulation of a target protein (*e.g.* ADP-GlcPPase) by thioredoxin. In the amyloplast, there is no photosynthetic electron transport, but like in other heterotrophic plastids, the oxidative pentose phosphate pathway generates reducing power. In the first two steps of this pathway, the Glc-6-P is oxidized producing NADPH by glucose-6-P dehydrogenase and 6-phosphogluconate dehydrogenase. In the amyloplast, the main carbon source, translocated from the cytosol, is either Glc-6-P or Glc-1-P (30). The NADPH produced in this light-independent process further reduces a ferredoxin form that is present in heterotrophic plastids and serve as reducing power for ferredoxin-dependent enzymes (21). It has been described that the cytosolic thioredoxin *h* is reduced by NADPH via a NADP-thioredoxin reductase as in bacteria (NADP-thioredoxin system). On the other hand, chloroplastic forms (*m* and *f*) use ferredoxin as an electron donor as mentioned above (ferredoxin-thioredoxin system). In heterotrophic plastids both electron donors are present, but it is not known whether either a ferredoxin-thioredoxin or NADP-thioredoxin system exists. More information about the biochemistry of the amyloplast is needed before we can draw any definite conclusion about the

role of reductive activation in the regulation the ADP-GlcPPase from potato tuber *in vivo*.

We postulate that in chloroplasts, where the reductive regulatory site (Cys^{12}) is conserved, reductive activation by thioredoxin could add fine-tuning to the allosteric effect of 3-PGA. In the amyloplast, demonstration of the presence of a thioredoxin-like molecule is required before further speculation on the physiological relevance of reductive regulation of the ADP-GlcPPase. To our knowledge, this is the first time that a non-allosteric mechanism of regulation is postulated in the synthesis of starch and that conditions in which an ADP-GlcPPase is activated by thioredoxin are reported.

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