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# Mechanism of Reductive Activation of Potato Tuber ADP-glucose Pyrophosphorylase*

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**The potato tuber (*Solanum tuberosum* L.) ADP-glucose pyrophosphorylase activity is activated by a incubation with ADP-glucose and dithiothreitol or by ATP, glucose- 1-phosphate, $Ca^{2+}$, and dithiothreitol. The activation was accompanied by the appearance of new sulfhydryl groups as determined with 5,5′-dithiobis(2-nitrobenzoic acid). By analyzing the activated and nonactivated enzymes on SDS-polyacrylamide gel electrophoresis under nonreducing conditions, it was found that an intermolecular disulfide bridge between the small subunits of the potato tuber enzyme was reduced during the activation. Further experiments showed that the activation was mediated via a slow reduction and subsequent rapid conformational change induced by ADP-glucose. The activation process could be reversed by oxidation with 5,5′-dithiobis(2-nitrobenzoic acid). Incubation with ADP-glucose and dithiothreitol could reactivate the oxidized enzyme. Chemical modification experiments with [$^{14}$C]iodoacetic acid and 4-vinylpyridine determined that the intermolecular disulfide bridge was located between $Cys^{12}$ of the small subunits of the potato tuber enzyme. Mutation of $Cys^{12}$ in the small subunit into either Ala or Ser eliminated the requirement of DTT on the activation and prevented the formation of the intermolecular disulfide of the potato tuber enzyme. The mutants had instantaneous activation rates as the wild-type in the reduced state. A two-step activation model is proposed.**

ADP-glucose pyrophosphorylase (ADPGlc PPase)[1] (ATP:α-D-glucose-1-phosphate adenylyltransferase, EC 2.7.7.27) catalyzes the synthesis of ADP-glucose as shown below and is the first committed step toward starch synthesis.

$$\alpha\text{-Glucose-1-P} + \text{ATP} \xrightarrow{Mg^{2+}} \text{ADPGlc} + PP_i$$

REACTION 1

This enzyme plays a major regulatory role in the biosynthesis of glycogen in bacteria and starch in plants (1–4). The major allosteric activator of the plant ADPGlc PPase is 3-phosphoglycerate (3PGA), and the allosteric inhibitor is orthophosphate ($P_i$) (2, 5–8).

ADPGlc PPase from all sources is found to be a tetrameric protein. However, the enzyme from the enteric bacteria is homotetrameric (1–3), whereas the enzyme from higher plants is a heterotetramer composed of two different subunits (4, 9). The amino acid sequence of the small subunit of plant ADPGlc PPase is highly conserved (80–95%), but the amino acid sequence of the large subunit shows less conservation (55–65%; Ref. 9).

The molecular masses of the small and large subunit of the enzyme from potato tuber were found to be 50 and 51 kDa, respectively (10). Two cDNAs encoding the large subunit and small subunit of the potato tuber ADPGlc PPase have been expressed in *Escherichia coli* (11, 12). The properties of the recombinant enzyme are very similar to those of the enzyme purified from potato tuber (12).

The activity of several chloroplast enzymes is regulated by reversible thiol/disulfide interchange (13, 14). During photosynthetic electron transport in the light, covalent redox modification is mediated by a redox chain, the ferredoxin-thioredoxin system, leading to reductive activation of several stromal target enzymes, *e.g.* fructose-1,6-bisphosphatase, NADP-malate dehydrogenase, phosphoribulokinase, etc. (15). However, little information is known on the possible activation of ADPGlc PPases via reduction. The activity of potato tuber ADPGlc PPase was found to be stimulated by dithiothreitol (DTT) (7, 16). The authors suggest the presence of key sulfhydryl (-SH) groups at the catalytic and/or allosteric site. The mechanism of DTT stimulation, however, is not known. In the current study, we present evidence that the activation is due to synergism involving the enzyme interacting with both DTT and its substrates. A reduction of the intermolecular disulfide bridge between $Cys^{12}$ of the two small subunits of potato tuber ADPGlc PPase is involved in the activation process.

## MATERIALS AND METHODS

### *Reagents*

ATP, ADPGlc, Glc-1-P, 3PGA, inorganic pyrophosphate, and 4-vinylpyridine were purchased from Sigma. [$^{32}$P]$PP_i$ were purchased from NEN Life Science Products. [$^{14}$C]Glc-1-P and [1-$^{14}$C]iodoacetic acid were from ICN Pharmaceuticals, Inc. All other reagents were purchased at the highest available commercial grade.

### *Purification of Wild-type and Mutant Potato Tuber ADPGlc PPases*

The wild-type and mutant ADPGlc PPase cDNAs were expressed as described previously (17). The wild-type enzyme was purified to apparent homogeneity as estimated from about 4 μg of protein on SDS-PAGE. The mutants were purified as the wild-type enzyme except that the heat treatment and the hydrophobic chromatography steps were eliminated, and a second Mono Q chromatography step was added. In the second Mono Q step, protein elution was done with 50 mM Hepes, pH 8.0, 5 mM $MgCl_2$, 1 mM EDTA, and 20% sucrose with a linear gradient from 0.15 M to 0.3 M over 20 min. The mutant enzymes were purified to about 50% homogeneity as estimated by SDS-PAGE with about 3 μg of protein.

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[1] The abbreviations used are: ADPGlc PPase, ADP-glucose pyrophosphorylase; Glc-1-P, glucose 1-phosphate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; 3PGA, 3-phosphoglycerate; BSA, bovine serum albumin.

### *Assay of ADPGlc PPase*

*Assay I*—In the pyrophosphorolysis direction, enzyme activity was assayed according to the method of Morell *et al.* (18). The reaction mixture contained 80 mM glycylglycine, pH 8.0, 2 mM ADP-glucose, 5 mM $MgCl_2$, 3 mM DTT, 2 mM [$^{32}$P]$PP_i$ (1,000–2,000 cpm/nmol), 10 mM NaF, 200 $\mu$g/ml bovine serum albumin, and enzyme in a total volume of 250 $\mu$l. This assay was primarily used for measuring activity during enzyme purification steps.

*Assay II*—In the ADP-glucose synthesis direction, enzyme activity was measured at 37 °C according to the method of Preiss *et al.* (19). Enzyme activity was usually measured in this direction in the kinetic analyses unless otherwise indicated. The reaction mixture contained 100 mM Hepes-NaOH, pH 8.0, 0.5 or 1 mM [$^{14}$C]Glc-1-P (1,000–3,000 cpm/nmol), 1.5 mM ATP, 5 mM $MgCl_2$, 3 mM DTT, 200 $\mu$g/$\mu$l bovine serum albumin, 0.3 unit of inorganic pyrophosphatase, and enzyme in a final volume of 200 $\mu$l. The reaction time was 1 min unless otherwise indicated.

### *Reductive Activation of ADPGlc PPase*

The enzyme was activated with reaction mixture A (100 mM Hepes, pH 8.0, 2 mM ADPGlc, and 3 mM DTT), at 37 °C for 30 min. This condition was referred to as the activation condition. In the control, the enzyme was incubated with reaction mixture B (100 mM Hepes, pH 8.0, and 2 mM ADPGlc). This condition was referred to as the nonactivation condition.

### *Determination of Available Sulfhydryl Groups with DTNB*

To determine the available sulfhydryl groups of the activated and nonactivated potato tuber ADPGlc PPase, enzyme (36 $\mu$g) was incubated either under the activation or nonactivation conditions. Then samples were desalted into mixture C (100 mM Hepes, pH 8.0, 5 mM $MgCl_2$, 1 mM EDTA, and 2 mM ADPGlc) with a Bio-Spin 30 column (Bio-Rad, Hercules, CA). 0.48 mM DTNB was added to the desalted enzyme, and the $A_{412\ nm}$ was measured every 15 s with a Beckman spectrophotometer model DU680. An extinction coefficient of 13,600 $M^{-1}$ $cm^{-1}$ was used for measuring the DTNB reduction (20). A molecular mass of 202 kDa (12) was used to calculate the amount of enzyme used for the DTNB measurement. The desalting procedure efficiently removed the DTT from the samples as indicated by the fact that in the absence of enzyme both mixture A and mixture B gave the same absorbance reading after desalting. After removal of the DTT, the newly formed –SH groups of the activated enzyme were retained as determined by SDS-PAGE under nonreducing conditions. Furthermore, the activity of the activated and nonactivated enzyme was fully retained after desalting as determined by a comparison of the specific activity of the corresponding enzyme before the procedure.

### *Protein Assay*

Protein concentration was determined by the method of Smith *et al.* (21).

### *Determination of the Reduction and Activation Time Course*

Enzyme (48 $\mu$g) was incubated with mixture A at 37 °C in a final volume of 48 $\mu$l. Aliquots of 2.5 $\mu$l were withdrawn periodically for activity measurement in the synthesis direction. In parallel, aliquots of 4 $\mu$l were withdrawn and immediately mixed with 4 $\mu$l of 100 mM iodoacetamide to stop the reduction prior to SDS-PAGE analysis as described below. The protein contents of the 100-kDa band (small subunit dimer) and 50-kDa band from each sample were quantified by scanning the stained gels with a Molecular Dynamics Computing Densitometer. The reduction time course was also obtained by measuring the decrease of the protein content of the 100-kDa band with time.

SDS-PAGE and Immunoblot Analysis

SDS-PAGE was done as described by Laemmli (22) on 10% polyacrylamide gel. 2-Mercaptoethanol was not added to the protein samples under nonreducing condition. The nonactivated potato tuber ADPGlc PPase (8 $\mu$g) was separated in SDS-PAGE under nonreducing conditions and subsequently blotted to a ProBlott membrane (Applied Biosystems Inc.). After staining with Coomassie Blue R250, the 100-kDa band (small subunit dimer) and 50-kDa band were cut for sequencing as described previously (23).

Proteins were transferred to nitrocellulose membrane for immunoblot analysis, and treated with affinity-purified rabbit anti-spinach leaf ADPGlc PPase IgG. The antigen-antibody complex was visualized by treatment with alkaline phosphatase-linked goat anti-rabbit IgG, followed by staining with BM purple AP-substrate precipitating reagent (Boehringer Mannheim).

### *Determination of the Total Number of Disulfide Linkages*

Potato tuber ADPGlc PPase (17 $\mu$g) was incubated with either mixture A (activation condition) or mixture B (nonactivation condition). 8 M urea was added and the samples were incubated 15 min at 50 °C and then for 30 min at 37 °C. Samples were brought to room temperature, and 20 mM iodoacetamide was added to block all the exposed free thiols. After precipitation with 10% trichloroacetic acid, the pellets were washed three times and dissolved with 8 M urea in 0.4 M $NH_4HCO_3$. 1 mM DTT was added, and the reduction was continued at 50 °C for 15 min and then at 37 °C for 30 min. 12.5 mM [$^{14}$C]iodoacetic acid (8,240 cpm/nmol) was added, and the samples were incubated in the dark for 30 min at room temperature. The reaction was stopped by addition of 50 mM 2-mercaptoethanol. The proteins were precipitated and washed as before; the pellets were dissolved with 2% SDS in 100 mM Hepes, pH 8.0. Eight ml of scintillation liquid was added, and the samples were counted in a Packard liquid scintillation analyzer.

### *Identification of the Intermolecular Disulfide Bridge*

*Direct Labeling with [$^{14}$C]Iodoacetic Acid*—Potato tuber ADPGlc PPase (216 $\mu$g) was incubated either under activation or nonactivation conditions as described before. Then 15 mM [$^{14}$C]iodoacetic acid (8,240 cpm/nmol) was added and incubated for 1 h in the dark at room temperature. [$^{14}$C]Carboxymethylation was terminated by acidification with 10% trichloroacetic acid.

*Reverse Labeling*—Potato tuber ADPGlc PPase (68 $\mu$g) was incubated either under activation or nonactivation conditions as described before. 20 mM 4-vinylpyridine was added to the incubated solution to stop the DTT-dependent reduction of protein and to block exposed thiols. 8 M urea was added and the samples were incubated 30 min at 50 °C and then 1 h at 37 °C. Samples were brought to room temperature and incubated 2 h. After precipitation with 10% trichloroacetic acid, the pellets were washed four times and dissolved with 25 $\mu$l of 8 M urea in 0.4 M $NH_4HCO_3$; 5 $\mu$l of 24 mM DTT was added, and samples were incubated at 50 °C for 15 min and then 37 °C for 30 min. After cooling down to room temperature, 10 $\mu$l of 40 mM [$^{14}$C]iodoacetic acid (8,240 cpm/nmol) was added and the samples were incubated in the dark for 15 min. Carboxymethylation was stopped by addition of 16 $\mu$l of 100 mM DTT.

*4-Vinylpyridine Labeling*—Potato tuber ADPGlc PPase (17 $\mu$g) was incubated either under activation or nonactivation conditions in a final volume of 15 $\mu$l. Then 2.8 $\mu$l of 200 mM 4-vinylpyridine was added, and the samples were incubated at 37 °C for 3 h. The labeled proteins were stored at –20 °C prior to sequence analysis.

### *Trypsin Digestion*

Trypsin digestion was performed according to a described procedure (24) on the potato tuber ADPGlc PPase after direct labeling with [$^{14}$C]iodoacetic acid. For the tryptic digestion of the potato tuber enzyme after reverse labeling, water was added to adjust the final concentration of urea to 2 M and then L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin was added at a trypsin to ADPGlc PPase ratio of 1:50 (w/w). The digestion was allowed to proceed for 24 h at 37 °C. The reaction was terminated by freezing of the samples at –20 °C.

### *Purification of Labeled Peptides by HPLC*

The tryptic digests were applied to a $C_{18}$ Vydac RP column (4.6 mm × 250 mm). Peptides were eluted with a linear gradient formed by mixing solvent A (0.1% trifluoroacetic acid) and solvent B (90% acetonitrile, 0.1% trifluoroacetic acid). A 5 to 60% solvent B gradient was used at a flow rate of 1.0 ml/min over 117 min. The radioactive fractions were pooled and the acetonitrile was evaporated with a Speed-Vac prior to further separation. For further purification of the pooled radioactive fractions from direct and reverse labeling, a 135-min linear gradient (5–45% solvent B) and a 90-min linear gradient (5–30% solvent B) was used, respectively, at a flow rate of 40 $\mu$l/min. Both HPLC separations were performed on a microbore $C_{18}$ Vydac RP column (0.8 mm × 250 mm).

### *Sequence Determination*

Peptides from HPLC and whole protein samples were applied to a Procise (Applied Biosystems model 494A) automated sequencer for amino acid sequence analysis.

### *Site-directed Mutagenesis*

The mutant enzymes with Ser and Ala substitutions at residue 12 of the small subunit of potato tuber ADPGlc PPase were designated as $S_{C12S}L_{wt}$ and $S_{C12A}L_{wt}$, respectively. The $S_{C12S}L_{wt}$ mutant was obtained

by using the QuickChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA; Ref. 25); the $S_{C12A}L_{wt}$ mutant was obtained by sequential polymerase chain reaction steps (26). Then the *Nco*I-*Kpn*I fragment containing the Ala substitution was transferred into the expression vector. The sequence of the whole *Nco*I-*Kpn*I fragment was verified by double-strand sequencing. The oligonucleotides used for mutagenesis are shown below with the underlined bases introduced to replace the codon initially encoding $Cys^{12}$ in the small subunit.

$S_{C12S}L_{wt}$: 5′-CGCAGAATTCACAGACATCTCTAGACCCAGATGC-3′
3′-GCGTCTTAAGTGTCTGTAGATCTGGGTCTACG-5′

$S_{C12A}L_{wt}$: 5′-ATTCACAGACAGCTCTAGACCCAGATGCTAGCCGGAGT-3′
3′-TAAGTGTCTGTCGAGATCTGGGTCTACGATCGGCCTCA-5′

SEQUENCES 1 AND 2

## RESULTS

### *Reductive Activation of Potato Tuber ADPGlc PPase*

*Activation of Potato Tuber ADPGlc PPase by ADPGlc and DTT*—When measured in the absence of activator, 3PGA, the catalytic activity of potato tuber ADPGlc PPase was found to increase with time showing nonlinear kinetics. Various combinations of effectors were tested for their ability to activate the enzyme during a preincubation at 37 °C. As seen in Fig. 1*A*, both ADPGlc and DTT were required to give about 10-fold activation of the potato tuber enzyme. In the absence of DTT, ADPGlc could slightly activate the enzyme (close to 2-fold). However, when DTT was included in the preincubation mixture in the absence of ADPGlc, about 70% activity was lost after a 30 min preincubation. To further examine the effect of DTT, the rate of ADPGlc synthesis was measured in the presence or absence of DTT. When DTT was eliminated from the assay mixture, the enzyme was kept in a low activity form. The conversion to a high activity form only took place when DTT was present (data not shown). This may suggest that reduction of a disulfide bridge(s) is involved in the activation process.

*Activation of Potato Tuber ADPGlc PPase by ATP, Glc-1-P, $Ca^{2+}$, and DTT*—Different combinations of ATP, Glc-1-P, $Ca^{2+}$, and DTT were also tested for their effect on the activation of potato tuber ADPGlc PPase. DTT was included in all the combinations since it was required for the activation. Since catalysis would take place when the three effectors (ATP, Glc-1-P, and $Mg^{2+}$) were present together, $Ca^{2+}$ was used as a substitute for $Mg^{2+}$ to separate the activation process from catalysis. Experiments showed that $Ca^{2+}$ could replace $Mg^{2+}$ as a cofactor for the potato tuber ADPGlc PPase at about 1/10 of the rate seen with $Mg^{2+}$, and the apparent affinity of $Ca^{2+}$ for the enzyme ($S_{0.5}$ = 1.8 mM)[2] is similar to that of $Mg^{2+}$ ($S_{0.5}$ = 2.0 mM). These findings suggest that $Ca^{2+}$ binds to the same site as $Mg^{2+}$ but with lower catalytic efficiency. After preincubation, EGTA and $Mg^{2+}$ were added to start the assay. Since EGTA has a very high affinity for $Ca^{2+}$ and a very poor affinity for $Mg^{2+}$, $Ca^{2+}$ in the assay mixture was efficiently chelated and $Mg^{2+}$ would be the cation for the reaction. This metal exchange method was successfully used in the study of activation of chloroplast fructose-1,6-bisphosphatase (14, 27). As shown in Fig. 1*B*, the enzyme was only activated when all three effectors were present at the same time. With DTT present in cases where one of the other two effectors were not present, enzyme activity actually decreased from the control value in the 30-min preincubation.

Since 9.6 nmol of ADPGlc was produced when all three effectors were present in the preincubation (see Fig. 1*B* legend), another experiment was conducted to differentiate the activation from ADPGlc and that from ATP, Glc-1-P and $Ca^{2+}$ in the preincubation. Fig. 2 indicates that even before ADPGlc was produced in the preincubation (2–6 min), the enzyme was already activated. This demonstrates that ATP, Glc-1-P, and $Ca^{2+}$ could activate potato tuber ADPGlc PPase without prior formation of ADPGlc.

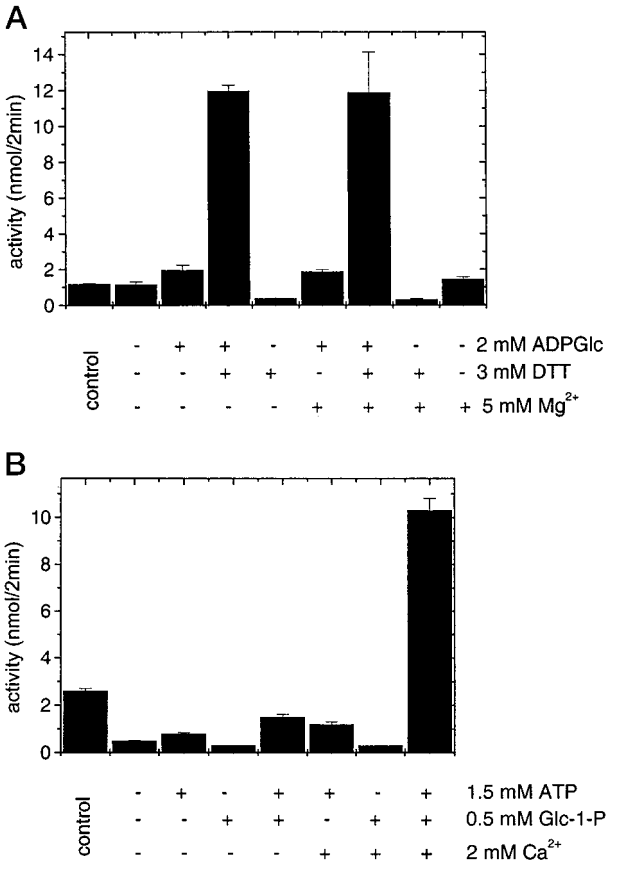


FIG. 1. *A*, activation of potato tuber ADPGlc PPase by ADPGlc and DTT. Enzyme (1.7 μg) was incubated with 100 mM Hepes, pH 8.0, 0.2 mg/ml BSA, and with different effectors in a final volume of 8 μl for 30 min at 37 °C. The synthesis reaction was started by adding 192 μl of assay mixture into the incubated solution and continued at 37 °C for 2 min. The control experiment was carried out without adding effectors, and the preincubation step was omitted. *B*, activation of potato tuber ADPGlc PPase by ATP, Glc-1-P, $Ca^{2+}$, and DTT. Enzyme (2 μg) was preincubated with a mixture that contained 80 mM glycylglycine, pH 8.0, 0.2 mg/ml BSA, 3 mM DTT and with different combinations of effectors in a final volume of 80 μl for 30 min. The synthesis reaction was started by adding 60 μl of incubated solution to 140 μl of assay mixture and continued at 37 °C for 2 min. A control experiment was carried out without adding effectors, and the activation step was omitted. Since the enzymatic reaction would slowly take place when the substrate components, ATP, Glc-1-P, and $Ca^{2+}$, were present in the preincubation, the value (10.3 nmol/2 min) was obtained after the subtraction of 9.6 nmol of ADPGlc produced in the 30-min preincubation. Whenever $Ca^{2+}$ was used, 1.6 mM EGTA was also included in the assay mixture.

*Reduction of an Intermolecular Disulfide Bridge during Activation*—To determine if a reduction occurred in the activation process, DTNB was used to quantitate the available sulfhydryl groups of the potato tuber enzyme under activated or nonactivated conditions. As shown in Fig. 3, the activation of potato tuber ADPGlc PPase was accompanied by an increase of about 2.5 new sulfhydryl groups per tetrameric enzyme over the nonactivated form. This difference correlates with the reduction of a disulfide bridge in the activation.

Since proteins with disulfide bridges often exhibit altered migration on SDS-PAGE under nonreducing conditions, both the activated and nonactivated potato tuber ADPGlc PPases were subjected to SDS-PAGE under nonreducing conditions (Fig. 4, *lanes a* and *b*). The activated protein migrated as a single band with molecular mass about 50 kDa (Fig. 4, *lane a*).

[2] Y. Fu and J. Preiss, unpublished results.

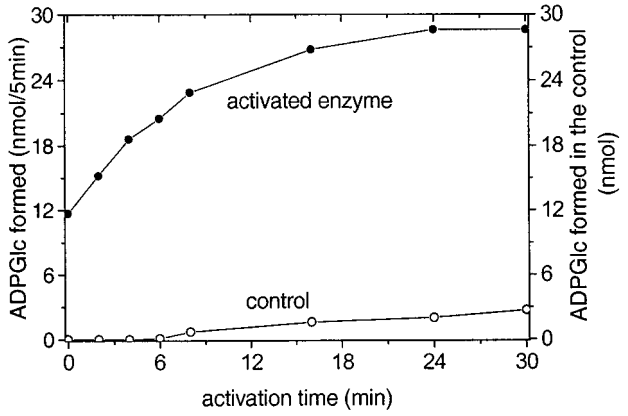


FIG. 2. **Activation of potato tuber ADPGlc PPase by ATP, Glc-1-P, $Ca^{2+}$, and DTT with different incubation times.** Enzyme (22.1 $\mu$g) was activated with a mixture containing 80 mM glycylglycine, pH 8.0, 0.2 mg/ml BSA, 3 mM DTT, 1.5 mM ATP, 2 mM $CaCl_2$, and 0.5 mM [$^{14}$C]Glc-1-P (942 cpm/nmol) at 37 °C in a final volume of 1,360 $\mu$l. An 80-$\mu$l aliquot was withdrawn periodically to measure the activity in the synthesis direction for 5 min. In parallel, an 80-$\mu$l aliquot was withdrawn to determine the amount of ADPGlc produced during activation as a control (○). The amount of product formed (●) for the incubated enzyme was obtained after the subtraction of the amount of ADPGlc produced during activation. 1.6 mM EGTA was included in the reaction mixture.

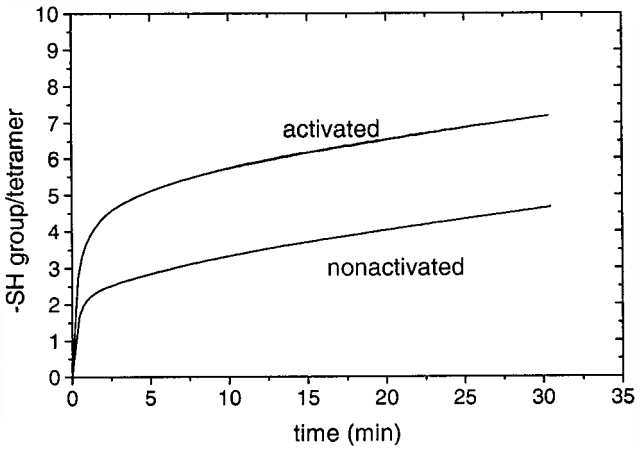


FIG. 3. **Determination of the available sulfhydryl groups of the activated and nonactivated potato tuber ADPGlc PPase with DTNB.** The experiment was performed as described under "Materials and Methods."

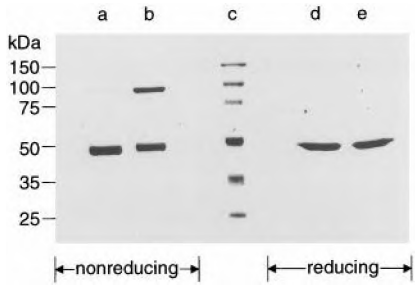


FIG. 4. **Electrophoretic analysis of the activated and nonactivated potato tuber ADPGlc PPase.** Enzyme (36 $\mu$g) was incubated under either activation or nonactivation conditions as described under "Materials and Methods." About 1.5 $\mu$g of activated (*lanes a* and *d*) or nonactivated enzymes (*lanes b* and *e*) were subjected to 10% SDS-PAGE under nonreducing (*lanes a* and *b*) or reducing (*lanes d* and *e*) conditions. The positions of molecular mass standards (*lane c*) are indicated.

This was in agreement with previous studies on native and cloned ADPGlc PPase (10, 12) that showed the molecular masses of the small and large subunit were 50 and 51 kDa,

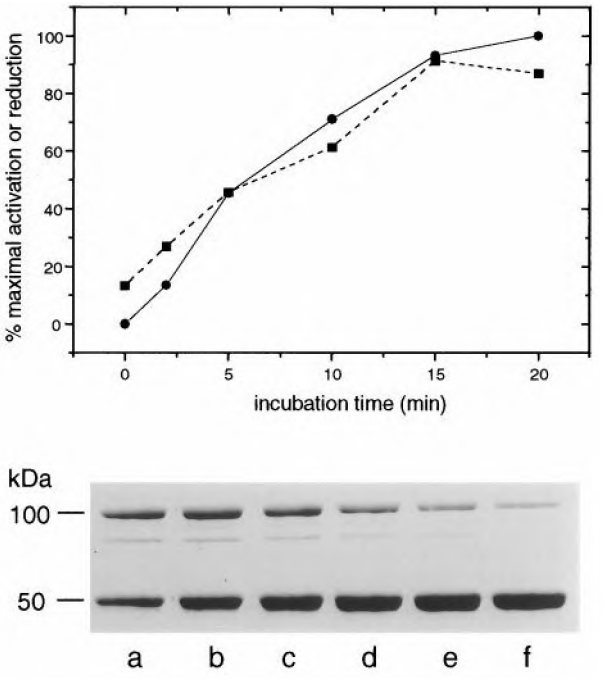


FIG. 5. **Time course of potato tuber ADPGlc PPase activation (●) and reduction (■).** Activation was performed in mixture A (100 mM Hepes, pH 8.0, 2 mM ADPGlc, and 3 mM DTT). Aliquots were withdrawn periodically for activity measurements and for SDS-PAGE analysis. The enzyme activities corresponding to 0 and 100% activation were 1.7 and 9.8 nmol/min, respectively.

respectively. Apparently, the small and large subunits in this electrophoresis system were too close to be distinguishable. The nonactivated enzyme migrated as two bands corresponding to molecular masses of 50 and 100 kDa (Fig. 4, *lane b*). Both bands were transferred to a ProBlott membrane, and their N-terminal sequences were determined. For the 100-kDa band, it was AVSDSQN; for the 50-kDa band, AVSVITT. The former was the same as the N-terminal sequence of the small subunit (12), and the latter was the same as that of the large subunit deduced from cDNA sequence (28) except that the first methionine was processed in both cases. Thus, the 100-kDa band was the dimer of the small subunit. This result indicated the existence of an intermolecular disulfide bridge between the small subunits of the potato tuber enzyme, which was reduced during activation. Under reducing conditions, both the activated and nonactivated enzyme migrated as a single band of 50 kDa (Fig. 4, *lanes d* and *e*).

*Time Course of Reduction and Activation*—Since a reduction step was involved in the activation of potato tuber ADPGlc PPase, it was of interest to compare the rate of reduction with the rate of activation. Because the reduction resulted in a shift of the dimer of the small subunit (100 kDa) to its monomer position (50 kDa) (Fig. 4), it was possible to determine the time course of the reduction by measuring the decrease of the protein content of the 100-kDa band with time. The results are shown in Fig. 5. The rate of enzyme activation matched closely the rate of reduction. It shows a correlation between the reduction and the activation process, suggesting that the reduction of the intermolecular disulfide is the rate-limiting step in the activation of potato tuber enzyme. A faint band just below the 100-kDa band was due to protein degradation from the small subunit during storage.

*Kinetics of Activation by ADPGlc and DTT*—As both ADPGlc and DTT were required for the activation of potato tuber ADPGlc PPase, an experiment was done to differentiate the effect of these two compounds. As shown in Fig. 6*A*, the potato tuber

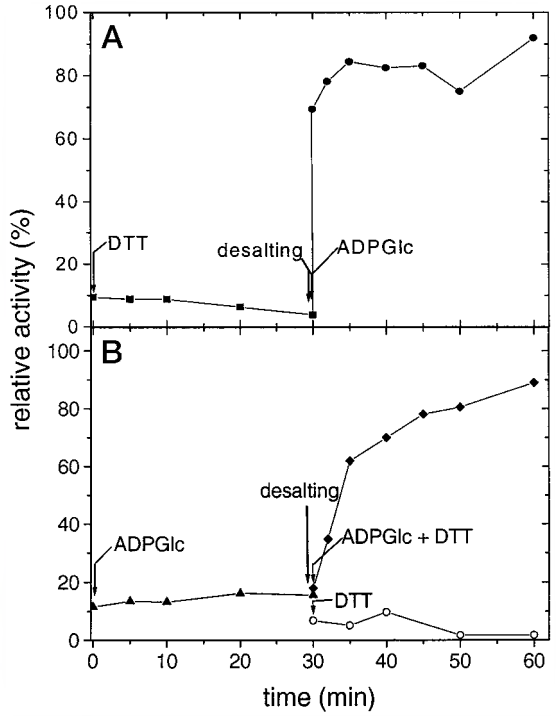


FIG. 6. **Kinetics of potato tuber ADPGlc PPase activation by ADPGlc and DTT.** In *panel A*, potato tuber enzyme (34 μg) was first incubated with 100 mM Hepes, pH 8.0, 0.2 mg/ml BSA, and 3 mM DTT (■) in a final volume of 160 μl at 37 °C. Aliquots of 2.5 μg of enzyme were withdrawn periodically to measure the activity in the synthesis direction. After 30 min, DTT was removed by desalting the enzyme rapidly into mixture D (100 mM Hepes, pH 8.0, 5 mM $MgCl_2$, and 1 mM EDTA). In the second incubation, the desalted enzyme was incubated with 100 mM Hepes, pH 8.0, 0.2 mg/ml BSA, and 2 mM ADPGlc (●) at 37 °C. The enzyme activity was measured at different times. In *panel B*, enzyme (42.5 μg) was first incubated with 100 mM Hepes, pH 8.0, and 2 mM ADPGlc (▲) in a final volume of 100 μl at 37 °C. Aliquots of 2.1 μg of enzyme were withdrawn periodically to measure activity as before. After 30 min, ADPGlc was removed by desalting the enzyme into mixture D. In the second incubation, 7/12 of the desalted enzyme was incubated with 100 mM Hepes, pH 8.0, 2 mM ADPGlc, and 3 mM DTT (◆); the rest was incubated with 100 mM Hepes, pH 8.0, and 3 mM DTT (○). Both incubations were done at 37 °C. The enzyme activity was measured at different incubation times. 100% activity corresponds to 16 nmol/min in *A* and 7.5 nmol/min in *B*.

enzyme was first incubated with DTT at 37 °C for 30 min, then DTT was removed by a rapid desalting step. Addition of ADPGlc resulted in instantaneous activation. The enzyme reached 80% of the maximal activity within 10 s. This is in agreement with the result in Fig. 5, which suggests that the reduction is the rate-limiting step of the activation process. During the first incubation with DTT, the enzyme activity decreased as seen in Fig. 1 (*A* and *B*). When the enzyme was first incubated with ADPGlc, subsequent addition of DTT could not activate the enzyme in the absence of ADPGlc (Fig. 6*B*). Still, both ADPGlc and DTT were required to activate the enzyme. The results suggest that reduction is a prerequisite for the activation.

In Table I, when both ADPGlc and DTT were removed from the incubation mixture, the activity of the activated enzyme decreased to below that of the nonactivated enzyme (control). A second incubation either with ADPGlc or with ADPGlc plus DTT reactivated the enzyme. Addition of DTT showed no effect in the reactivation process, indicating that the intermolecular disulfide bridge was already reduced. This was confirmed by SDS-PAGE analysis (under nonreducing condition), showing that the desalted enzyme remained reduced after the first incubation. The data indicate that the ADPGlc-induced conformational change of the reduced enzyme is reversible.

TABLE I
*Reactivation of potato tuber ADPGlc PPase*

In the first incubation, enzyme (25 μg) was incubated with 2 mM ADPGlc and 3 mM DTT at 37 °C for 30 min. Then the reagents were removed by desalting the enzyme into a solution containing 100 mM Hepes, pH 8.0, 5 mM $MgCl_2$, and 1 mM EDTA. In the second incubation, the desalted enzyme was incubated either with 2 mM ADPGlc or 2 mM ADPGlc plus 3 mM DTT at 37 °C for 30 min. 100 mM Hepes, pH 8.0, was included in all the incubations. Aliquots of 2.1 μg of enzyme were withdrawn after each treatment to measure the activity.

| Treatment | Activity |
|---|---|
| | *nmol/min* |
| Control | 1.1 ± 0.1 |
| First incubation | 13.6 ± 0.1 |
| Desalting | 0.7 ± 0.1 |
| Second incubation (ADPGlc) | 8.6 ± 0.4 |
| Second incubation (ADPGlc + DTT) | 8.7 ± 0.1 |

*Reversibility of the Reductive Activation*—When the activated (reduced) form (Fig. 7*A*, *lane b*) of enzyme was incubated with DTNB, a mobility shift of the 50-kDa band to the 100-kDa position could be observed on SDS-PAGE under nonreducing conditions (Fig. 7*A*, *lane c*). Oxidation by DTNB was accompanied by a decrease of the enzyme activity (Fig. 7*B*), suggesting the reformation of the intermolecular disulfide bridge. The DTNB-treated enzyme could be reactivated by a second preincubation with ADPGlc and DTT. Upon reactivation, the intermolecular disulfide bridge was reduced again (Fig. 7*A*, *lane d*).

*Identification of the Intermolecular Disulfide Bridge*—There are 28 cysteine residues in potato tuber ADPGlc PPase (6 in the small subunit and 8 in the large subunit). Prior to locating the intermolecular disulfide bridge between the small subunits, the total number of disulfide bridges of potato tuber ADPGlc PPase was determined by [$^{14}$C]iodoacetic acid labeling. The nonactivated (oxidized) enzyme was first denatured with urea to expose all free sulfhydryl groups, which were blocked by subsequent addition of iodoacetamide. Then the protein was reduced with DTT before being labeled with [$^{14}$C]iodoacetic acid. In this way, only the oxidized (disulfide) groups would be labeled. It was found that 2.7 -SH were labeled per tetrameric protein. When this procedure was applied to the activated enzyme, 0.7 -SH was labeled per tetrameric protein, apparently from nonspecific labeling. This indicates that there is only one disulfide bridge in the potato tuber ADPGlc PPase.

To determine the location of the intermolecular disulfide bridge, both the activated and nonactivated enzyme were labeled with [$^{14}$C]iodoacetic acid and then digested with trypsin. The digests were separated by reversed-phase HPLC. As shown in Fig. 8*A*, one major radioactive fraction (*peak A*, 60% of total radioactivity) was obtained for the activated enzyme. After further purification by HPLC, sequence analysis showed that its N-terminal sequence corresponded to Ala$^2$–Ser$^{18}$ in the small subunit of potato tuber ADPGlc PPase (A in Table II). One labeled carboxymethylcysteine was identified at cycle 11. For the nonactivated enzyme, the overall labeling was low (data not shown), suggesting most sulfhydryl groups were buried in the protein. The sequences for three other minor radioactive peaks were not determined due to their low level of labeling (Fig. 8*A*).

To eliminate the possibility that the labeling of A was due to the unmasking of buried sulfhydryl groups in the activated enzyme, a reverse labeling experiment (see "Materials and Methods"), which would specifically label the oxidized (disulfide) groups, was performed for the nonactivated enzyme. As shown in Fig. 8*B*, only one major radioactive peak was obtained. Its N-terminal sequence was determined after further HPLC separation. The major sequence corresponded to Ala$^2$–Pro$^{15}$ in the small subunit (B in Table II). One carboxymethyl-

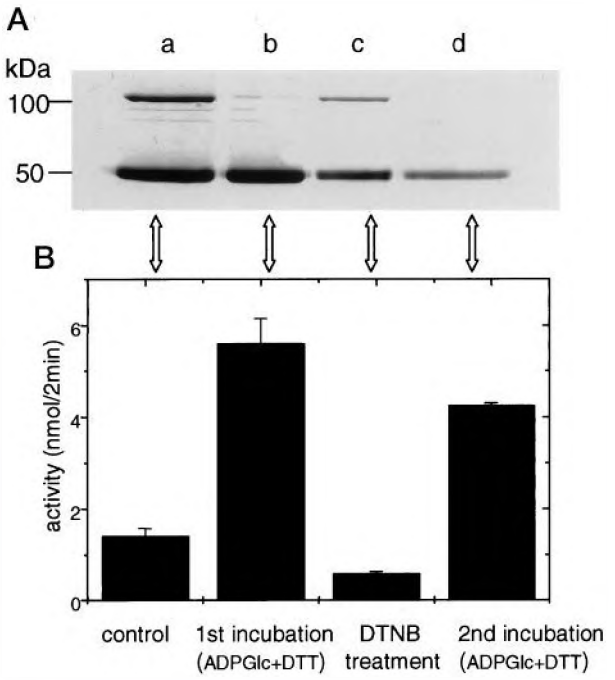


FIG. 7. **Reversibility of the reductive activation of potato tuber ADPGlc PPase.** In the control, 38 $\mu$g of enzyme was mixed with 100 mM Hepes, pH 8.0 and 2 mM ADPGlc in a final volume of 80 $\mu$l. 2 $\mu$l of 100 mM DTT was added to initiate the first incubation. After 30 min at 37 °C, DTT was removed by desalting the enzyme into mixture C (100 mM Hepes, pH 8.0, 5 mM $MgCl_2$, 1 mM EDTA, and 2 mM ADPGlc). Then 2 mM DTNB was added and the oxidation was allowed to proceed at room temperature for 30 min. DTNB was removed by desalting as before. Reactivation was started by incubating the desalted enzyme with 2 mM ADPGlc and 3 mM DTT in a final volume of 45 $\mu$l at 37 °C for 30 min. Aliquots of about 1.2 $\mu$g of enzyme were withdrawn at different times to measure the activity in the synthesis direction (*B*). In parallel, aliquots of about 4.8 $\mu$g of enzyme were withdrawn for SDS-PAGE analysis under nonreducing condition (*A*).

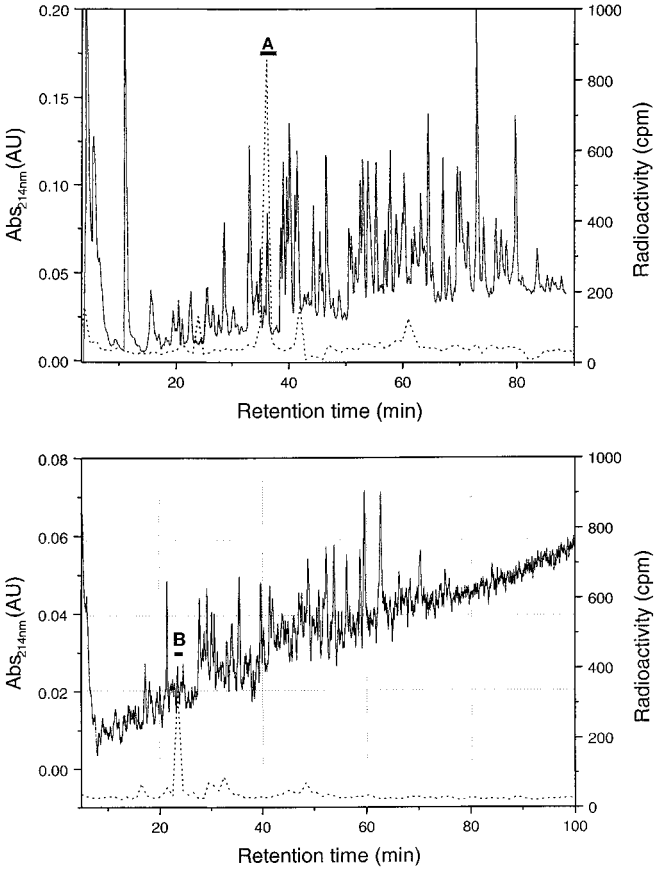


FIG. 8. *A*, HPLC separation of tryptic fragments from the activated and $^{14}$C-carboxymethylated potato tuber ADPGlc PPase. Elution was monitored by the UV absorption at 214 nm (*solid line*), and the radioactivity was determined by withdrawing a 200-$\mu$l aliquot from each fraction and measuring by liquid scintillation counting (*dotted line*). Fractions subjected to further HPLC purification for the purpose of Edman degradation are indicated by *bars* and *capital letters*, with further details provided in Table II. *B*, HPLC separation of tryptic fragments of the nonactivated potato tuber ADPGlc PPase after reverse labeling with [$^{14}$C]iodoacetic acid. The conditions are the same as described in *A*.

cysteine was identified at cycle 11. This is in agreement with the result from Fig. 8*A*. There was also a minor sequence present in *B* corresponding to $Ala^{196}$–$Lys^{208}$ in the small subunit. It did not contain any cysteine residue. When this procedure was performed on the activated enzyme, no significant labeling could be observed (data not shown).

In order to avoid any ambiguity, sequence analysis was performed on 4-vinylpyridine labeled whole protein since the N-terminal cysteines of the small subunits were implicated in forming the disulfide bridge. The result shows two sequences corresponding to both the small and large subunit. Based on the known sequences of the two subunits, the sequence of each subunit could be deduced. As shown in Table II, $Cys^{12}$ in the small subunit of the activated enzyme was labeled by 4-vinylpyridine. In contrast, sequence analysis did not detect any residue at the same position in the small subunit of the nonactivated enzyme, suggesting the presence of nonderivatizable sulfhydryls (unmodified cysteine is not stable during Edman degradation). The data confirm the results obtained by direct labeling (A in Table II) and reverse labeling (B in Table II) by [$^{14}$C]iodoacetic acid. These results demonstrate that the cysteine residues located at position 12 of the two small subunits are linked together by a disulfide bridge in the nonactivated (oxidized) potato tuber ADPGlc PPase. The disulfide linkage was reduced during the activation.

*Production and Purification of Mutant Enzymes*—The expression of mutant ADPGlc PPase cDNAs was confirmed by resolving the crude extract proteins on SDS-PAGE. Potato tuber ADPGlc PPases were identified by immunoblotting with antibody against spinach leaf ADPGlc PPase that has been shown to be reactive with the potato tuber enzyme (10). The two mutant enzymes, $S_{C12S}L_{wt}$ and $S_{C12A}L_{wt}$, were produced at level similar to the wild-type enzyme based on the intensity of the immunoblotting. Their apparent sizes were the same as that of the wild-type.

To determine if the mutations prevented the formation of the intermolecular disulfide between the small subunits, the mutant and wild-type enzymes were subjected to SDS-PAGE under reducing and nonreducing conditions and transferred to nitrocellulose membranes. Immunoblotting results showed that the mutant proteins migrated as a single band under nonreducing condition, while the wild-type migrated as two bands corresponding to molecular masses of 50 and 100 kDa (Fig. 9). Under reducing conditions, the mutant and wild-type enzymes migrated as a single band. Thus, mutagenesis indeed eliminated the intermolecular disulfide in potato tuber enzyme. This observation confirms the results obtained from chemical modification approaches.

*Activation Characteristics of Mutant Enzymes*—As shown in Fig. 10, substitution of $Cys^{12}$ in the small subunit by either Ser or Ala eliminated the requirement of DTT for the activation of the potato tuber ADPGlc PPase. Another striking difference between the mutant and wild-type enzymes was the time course of activation. The wild-type needed 17.5 min to reach maximal activity, the two mutant enzymes were fully activated

TABLE II
*Sequence analysis of [$^{14}$C]iodoacetic acid-labeled tryptic peptides and 4-vinylpyridine-labeled ADPGlc PPases*

A and B refer to purified labeled fractions of the HPLC chromatograms of Fig. 8 (A and B). C and D refer to the activated and nonactivated and 4-vinylpyridine-labeled potato tuber ADPGlc PPases, respectively. Both C and D contain two N-terminal sequences corresponding to the small and large subunits. The sequence corresponding to each subunit was deduced from the known sequences of the two subunits (12, 28). Italic sequences were assigned to the small subunit. The residue number of the first amino acid of peptide A refers to the sequence of the small subunit (12). The labeled cysteine residues were underlined: Cm-C, carboxymethylcysteine; PE-C, *s*-b-(4-pyridylethyl)-cysteine. Cm-C and PE-C were identified by comparison with the elution times of the two standard cysteine derivatives as determined separately.

| Peptides or proteins | Amino acid sequences |
|---|---|
| | 2 |
| A | *AVSDSQNSQTCm-CLDPDAS* |
| B[a] | *AVSDSQNSQTCm-CLDP* |
| C | *AVSDSQNSQTPE-CLD*<br>AYSVITTENDT QT |
| D | *AVSDSQNSQTX*[b] L<br>AYSVITTENDTQ |

[a] Besides the major sequence shown in the table, there was also a minor sequence corresponding to Ala$^{196}$–Lys$^{208}$ in the small subunit.
[b] *X* indicates that no amino acid could be detected in the cycle.

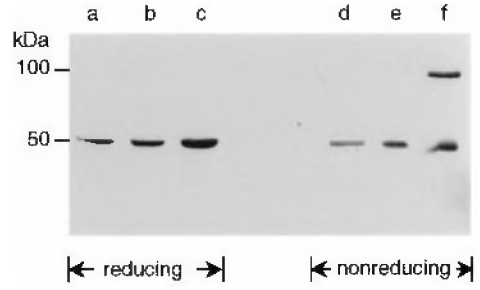


FIG. 9. **Immunoblot analysis of wild-type and mutant potato tuber ADPGlc PPases.** Wild-type (*lanes c* and *f*), $S_{C12S}L_{wt}$ (*lanes a* and *d*), and $S_{C12A}L_{wt}$ (*lanes b* and *e*) were subjected to SDS-PAGE under reducing (*lanes a–c*) and nonreducing (*lanes d–f*) conditions and transferred to nitrocellulose membranes. Immunoreactive species were detected using antibody against spinach leaf ADPGlc PPase.

within 10 s. Thus, the mutant enzymes show the same activation characteristics as the reduced wild-type (Fig. 6*A*).

## DISCUSSION

The present study shows that the activation of the potato tuber ADPGlc PPase proceeds via a reduction of the intermolecular disulfide bridge between the small subunits and a subsequent conformational change induced by the substrates. The observation that all three ligands, ATP, Glc-1-P, and $Ca^{2+}$ ($Mg^{2+}$), are required to be present to have an equivalent activation effect as ADPGlc on the enzyme is consistent with an ordered binding mechanism as previously shown for ADPGlc PPases from *E. coli* (29), *Rhodospirillum rubrum* (30), and barley leaf (31). ATP:$Mg^{2+}$ binds first, and then Glc-1-P binds. $Mg^{2+}$ was required for the binding of ATP, but not for the binding of ADPGlc (29). Therefore, all substrates, ATP, Glc-1-P, and $Ca^{2+}$ ($Mg^{2+}$), are needed to bind the catalytic sites in contrast to ADPGlc. In this regard, it seems that both the ATP site and Glc-1-P site are required to be occupied in order to induce the conformational change following the reduction step.

Reduction of an intermolecular disulfide bridge resulted in a shift of the dimer band of the small subunit to its monomer position in SDS gels. By analyzing protein samples withdrawn from different time points of the activation, the reduction time course could be visualized. This technique can be conveniently used to follow the reduction course of intermolecular disulfide bridges in proteins. Both Figs. 5 and 6 indicate that the activation of the potato ADPGlc PPase is mediated via a slow reduction and a rapid conformational change. Moreover, the enzyme needs to be reduced first in order for the conformational change to take place. For some enzymes such as chloroplast fructose-1,6-bisphosphatase, the rate of the reduction process is strongly accelerated by specific conformational changes induced by modulators (32). However, inclusion of ADPGlc did not show acceleration of the reduction rate of potato tuber ADPGlc PPase (data not shown). This further indicates that the activation of potato tuber enzyme proceeds in

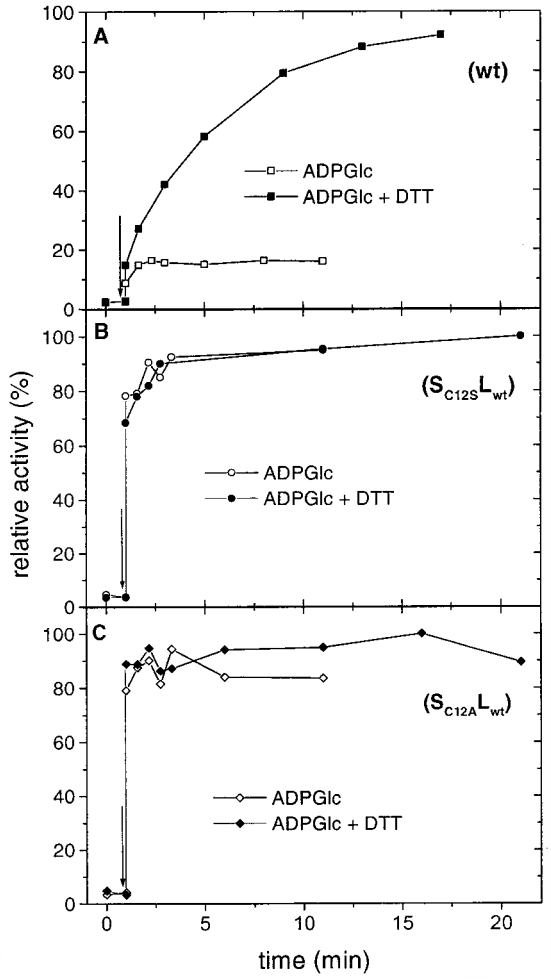


FIG. 10. **Activation kinetics of the wild-type (*wt*) and mutant potato tuber ADPGlc PPases.** The untreated enzymes were incubated at 37 °C for 1 min, then the activation medium (2 mM ADPGlc or 2 mM ADPGlc + 3 mM DTT) was added and the activity was measured in the synthesis direction on aliquots of 20 $\mu$l at various times. 100% activity of the wild-type, $S_{C12S}L_{wt}$, and $S_{C12A}L_{wt}$ enzymes correspond to 16.6, 8.7, and 10.3 nmol/min, respectively. *Arrows* indicated the addition of ADPGlc or ADPGlc plus DTT.

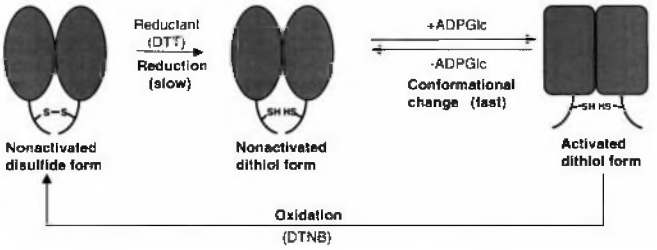


FIG. 11. **A proposed model of the reductive activation mechanism of potato tuber ADPGlc PPase.** Only the small subunits are shown. The N-terminal extensions are shown for convenience, on the outside of each subunit.

a sequential order with the reduction occurring first.

When used to measure the sulfhydryl groups in proteins, DTNB can result in formation of disulfides in proteins (20). DTNB treatment could reverse the activation of potato tuber ADPGlc PPase by reoxidizing the reduced intermolecular disulfide bridge (Fig. 7). Addition of ADPGlc and DTT reactivated the enzyme. These results suggest that the activated dithiol form and the nonactivated disulfide form of the enzyme could be interconverted. The intermolecular disulfide bridge seems to act as a regulator for the activation process. Although DTNB treatment resulted in formation of disulfide in ADPGlc PPase, the stoichiometry that 1 mol of 2-nitro-5-thiobenzoate anion was formed/mol of protein sulfhydryl still applied.

It is important to distinguish the activation induced by the substrates plus DTT from that induced by the physiological activator, 3PGA. All the studies on the reductive activation of potato tuber ADPGlc PPase were performed in the absence of 3PGA. When 3PGA was included in the reaction mixture, the enzyme showed linear kinetics and the specific activity was about 13-fold higher in the ADPGlc synthesis reaction and 2.5-fold higher in the pyrophosphorolysis reaction than that from the reductive activation.

Intermolecular disulfide bridges are often involved in maintaining the quaternary structure of proteins. Besides its involvement in the activation of potato tuber ADPGlc PPase, the intermolecular disulfide bridge between the small subunits is apparently also involved in maintaining the enzyme in a correct folding state. The potato tuber ADPGlc PPase became unstable when DTT was present in the preincubation mixture (Fig. 1, *A* and *B*). Once the disulfide bridge was reduced, either ADPGlc or ATP, Glc-1-P plus $Ca^{2+}$ are needed to protect the enzyme from inactivation. Consistent with this observation is that reduced wild-type and mutant enzymes are heat-labile at 60 °C for 5 min, while the wild-type enzyme is stable at this condition (12).

Mutation of $Cys^{12}$ in the small subunit into either Ala or Ser yielded mutants with instantaneous activation rates as the wild-type in the reduced state. This suggested that the role of $Cys^{12}$ was neither related to its hydrophobicity nor its hydrogen-bonding capacity but specifically to its ability to form a disulfide bridge. Sequence alignment of all the plant ADPGlc PPases available indicates that $Cys^{12}$ and its surrounding sequence, -S-Q-T-C-L-D-P-, is conserved in the small subunit of enzymes from all dicot plants, *e.g.* spinach leaf, *Vicia faba*, *Beta vulgaris*, *Pisum sativum*, *Ababidopsis thaliana,* etc. It is also conserved in the small subunit of one monocot plant enzyme, that from barley leaf. [3] However, ADPGlc PPase from spinach leaf could not be activated by ADPGlc and DTT. By analyzing the spinach leaf ADPGlc PPase on SDS-PAGE under nonreducing condition, it was also found that an intermolecular disulfide bridge existed between its small subunits.[2] Reduction of this disulfide bridge made the spinach leaf enzyme heat labile as in the case of the potato tuber enzyme. Information regarding the reductive activation of the other ADPGlc PPases with the conserved Cys is not available.

Several chloroplast enzymes are regulated by reversible thiol-disulfide interchange mediated by light controlled ferredoxin-thioredoxin system (14). Interestingly, the same potato ADPGlc PPase small subunit gene is expressed both in tubers (non-photosynthetic tissue) and leaves (photosynthetic tissue; Ref. 33). However, the expression level in leaves is significantly lower than that in tubers. It is not clear if the same potato tuber ADPGlc PPase is also expressed in potato leaves. When reduced thioredoxin from *Spirulina* was substituted for DTT to activate the potato tuber ADPGlc PPase, no significant effect could be observed. There is still a possibility that a different isozyme of thioredoxin may be active. However, the physiological importance of the reductive activation phenomenon in this enzyme is still unclear as, *in vivo*, the enzyme may be continuously exposed to the activator 3PGA. Nevertheless, the possibility cannot be discarded that an indigenous reductant plays a role in the fine regulation of the potato tuber enzyme.

A proposed activation mechanism of potato tuber ADPGlc PPase is shown in Fig. 11. The intermolecular disulfide bridge between the small subunits locks the protein in a nonactivated conformation. Reduction frees the enzyme, and subsequent binding of ADPGlc induces a rapid conformational change of the enzyme to the activated state. Removal of ADPGlc converts the enzyme back to its nonactivated dithiol form, while reoxidation of the intermolecular disulfide bridge converted the enzyme back to its nonactivated disulfide conformation. For clarity, only the small subunits are shown, but it must be kept in mind that the reduction of the intermolecular disulfide bridge probably leads to a rearrangement of the small and large subunits during the activation.

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[3] B. Smith-White and J. Preiss, unpublished results.

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