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THE GLUCOSE-1-PHOSPHATE BINDING SITE AND
THE MECHANISM OF REDUCTIVE ACTIVATION OF
POTATO TUBER ADP-GLUCOSE PYROPHOSPHORYLASE

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Jack Preiss
Major professor

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By

YINGBIN FU

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ABSTRACT

THE GLUCOSE-1-PHOSPHATE BINDING SITE AND THE MECHANISM OF REDUCTIVE ACTIVATION OF POTATO TUBER ADP-GLUCOSE PYROPHOSPHORYLASE

By

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ADP-glucose pyrophosphorylase (ADPGlc PPase) from higher plants is a heterotetramer composed of two different subunits. The expression of a putative full-length cDNA encoding the small subunit of potato tuber ADPGlc PPase in *Escherichia coli* results in a catalytically active enzyme. Its specific activity is similar to that of the purified holoenzyme when assayed at a saturating concentration of activator. The activity of the large subunit is negligible. The homotetrameric small subunit differs from the heterotetrameric enzyme only in its regulatory properties. Thus, the small subunit is proposed to be mainly involved in catalysis, while the large subunit is mainly involved in modulating the regulatory properties of the small subunit. The N-terminus of the small subunit is most likely involved in the regulatory properties and heat stability of the holoenzyme since these properties were shown previously to be altered in a recombinant enzyme that contains the truncated small subunit (at the N-terminus) and the large subunit.

Lys^{195} in the homotetrameric ADPGlc PPase from *E. coli* was shown to be involved in the binding of the substrate, glucose-1-phosphate in a previous study [Hill, M. A., Kaufmann, K., Otero, J., and Preiss, J. (1991) *J. Biol. Chem.* **266**, 12455-12460]. This residue is highly conserved in the ADPGlc PPases. Replacing the conserved Lys^{198} of the small subunit of the potato tuber enzyme with arginine, alanine, and glutamic acid decreased the apparent affinity for glucose-1-phosphate of the wild-type enzyme 135- to 550-fold. Mutagenesis of the conserved Lys^{213} with arginine, alanine or glutamic acid in the large subunit had no effect on the apparent affinity for glucose-1-phosphate. A double

mutant, $S_{K198R}L_{K213R}$, had a 100-fold lower apparent affinity for glucose-1-phosphate. These findings indicate that Lys^{198} in the small subunit is directly involved in the binding of glucose-1-phosphate, while Lys^{213} in the large subunit has no effect on the apparent affinity for the sugar phosphate substrate.

The catalytic activity of potato tuber enzyme is activated by a preincubation with ADP-glucose and dithiothreitol or by ATP, glucose-1-phosphate, Ca^{2+}, and dithiothreitol. Using 5, 5'-dithiobis (2-nitrobenzoic acid) quantitation and SDS-PAGE analysis, it was shown 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 is reversible by interchanging of the enzyme between its activated dithiol form and the nonactivated disulfide form. The intermolecular disulfide bridge was identified to be located between the Cys^{12} residues of the small subunits of the potato tuber enzyme by chemical derivatization and site-directed mutagenesis approaches. This reductive activation mechanism is explained by a two-step model.

To my family

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LIST OF ABBREVIATIONS

ADPGlc PPase	ADP-glucose pyrophosphorylase
Glc-1-P	glucose-1-phosphate
DTNB	5, 5'-dithiobis (2-nitrobenzoic acid)
DTT	dithiothreitol
FBP	fructose-1, 6-bisphosphate
Man-1-P	mannose-1-phosphate
Pi	orthophosphate
PLP	pyridoxal 5′-P
PMI/GMP	phosphomannose isomerase-guanosine 5′-diphospho-D-mannose pyrophosphorylase
PPi	pyrophosphate
TCA	trichloroacetic acid
3PGA	3-phosphoglycerate
6F-Glc-1-P	6-fluoro-glucose-1-phosphate

INTRODUCTION

Starch is an end product of carbon fixation and the major storage form of photosynthate in the sink tissues of many crop plants. Research on the biosynthetic pathway of starch not only can lead toward understanding one of the most basic anabolic pathways but also provides the potential to manipulate the production and structure of starch for many applications in agriculture as well as industries.

The ADP-glucose pathway is believed to be the predominant route towards starch synthesis (1, 2). ADP-glucose pyrophosphorylase (ADPGlc PPase, EC 2.7.7.27) catalyzes the synthesis of ADP-glucose from ATP and glucose-1-phosphate (Glc-1-P) and is the major regulatory enzyme in the starch biosynthetic pathway (1- 5).

The goal of this thesis project is to identify important residues or domains in the catalysis and regulation of the potato tuber ADPGlc PPase. Chapter 1 is a review of literature that provides information about the physiological role and the structure-function studies on ADPGlc PPases with the emphasis on plant enzymes.

Potato (*Solanum tuberosum L.*) tuber ADPGlc PPase has two different subunits— the small and large subunits (6), both of which were cloned (7). Before I began to work on this project, the cloned cDNAs were expressed together in *Escherichia coli* (8). Some of the properties of the recombinant enzyme were different from the native enzyme and were ascribed to an N-terminal truncation of the small subunit (8). In the work presented in Chapter 2, the expression of an extended cDNA of the small subunit that includes those truncated amino acids in *E. coli* yielded a homotetrameric enzyme with a specific activity comparable to that of the heterotetrameric enzyme consisting of the small and large subunits. The properties of the homotetrameric and heterotetrameric enzymes are compared in detail. The roles of the small and large subunits in plant ADPGlc PPases are discussed. The properties of the heterotetrameric enzyme containing the large and extended small subunits are more similar to that of the native enzyme than the heterotetrameric enzyme, which contained the large and truncated small subunits. This work was done in collaboration with Dr. Miguel A. Ballicora. Part of the data were published in *Plant Physiol.* (9).

In ADPGlc PPase from *E. coli*, Lys^{195} has been identified as part of the binding site for Glc-1-P (10, 11). This residue is highly conserved in the homotetrameric bacterial enzymes as well as in both the small and large subunits of heterotetrameric plant ADPGlc PPases (4, 12). However, no study had been performed to characterize these conserved lysine residues in the plant enzyme. The expression system for potato tuber enzyme in *E. coli* provided a useful tool to serve this purpose. This study was also helpful in further clarifying the roles of the two subunits since recent studies suggest that the small and large subunits of the potato tuber enzyme cannot simply be classified as catalytic and regulatory subunits (13), respectively. Chapter 3 describes the site-directed mutagenesis studies on the putative Glc-1-P binding sites, Lys^{198} on the small subunit and Lys^{213} on the large subunit, of the potato tuber enzyme (14).

Chapter 4 deals with the reductive activation mechanism of the potato tuber ADPGlc PPase. It started from the observation that the catalytic activity of potato tuber enzyme increases with time (showing nonlinear kinetics) when measured in the absence of the activator. Further experiments suggested that the reduction of a disulfide bridge(s) might be involved in the activation. Although metabolic regulation by means of thiol/disulfide exchange has been well established in the light regulation of photosynthetic enzymes in the chloroplast via thioredoxin/ferredoxin system (15, 16), redox modulation was not observed with any of the ADPGlc PPases. The potato tuber ADPGlc PPase was shown to be activated by dithiothreitol (DTT) (17, 18), but the mechanism was not understood. In Chapter 4 I provide various evidence that the activation is due to a reduction of an intermolecular disulfide bridge between two small subunits followed by a conformational change induced by substrates. The intermolecular disulfide bridge is identified by both chemical derivatization and site-directed mutagenesis approaches. This work was submitted for publication in *J. Biol. Chem.*

Finally, Chapter 5 summarizes my thesis project and discusses the perspectives for future study.

Yingbin Fu

East Lansing, Michigan

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CHAPTER 1

LITERATURE REVIEW

LITERATURE REVIEW

1. General Properties and Structure of Starch

1.1. Properties

Starch is a white, granular or powdery, odorless, tasteless, complex carbohydrate. It is almost insoluble in cold water and in alcohol, but forms a colloidal suspension in boiling water. Starch is an end product of carbon fixation by photosynthesis and is a major reserve polysaccharide of green plants. It is almost universally present in every type of plant tissues, e.g., leaves, fruits, pollen grains, roots, etc., but especially abundant in the seeds of cereal plants and in bulbs and tubers.

1.2. Structure

Starch is organized within a three-dimensional, semicrystalline structure named "starch granule". The starch granules are formed in plastids—mainly the amyloplast and the chloroplast. Amyloplast is developed from proplastids and specialized in the synthesis and long-term storage of starch in nonphotosynthetic plant tissues. In the chloroplast, starch serves as a temporary storage of energy and carbon for photosynthetic cells. In leaves, starch is synthesized in the chloroplasts during the day from photosynthetically fixed carbon and is degraded by respiration at night. The major site of starch accumulation is in storage organs including seeds, fruits, tubers, and storage roots. Starch content can range between 65% and 90% of the total dry weight in those organs.

Starch contains two kinds of polysaccharides: amylose and amylopectin. Amylose consists of linear chains of α-(1$\rightarrow$4)-linked glucose residues with molecular weight around 2.5×10^5. It constitutes about 20% of ordinary starch, though this number can vary between 11 and 36% (1). In contrast to amylose, amylopectin is highly branched with an average of one α-(1$\rightarrow$6) glucosidic linkage every 20 to 26 glucosyl residues (2, 3). Amylopectin usually constitutes about 70% of the starch granule. Its average molecular weight is around 10^8. Some amylopectins, notably those from potato tuber, are also phosphorylated. It has been reported that up to one phosphate per 300 glucose residues was found in potato tuber amylopectins (4). Besides amylose and amylopectin, proteins (including the enzymes of starch biosynthesis) and lipid constitutes the other minor components of the starch granules.

1.3. Application

Besides its main usage as food in the world, about one third of the total production of starch is used for many industrial processes, e.g., sizing of paper and board, packaging, manufacture of biodegradable polymers, textile industries, etc. In the chemical industry, starch is used as a raw material to produce acids, polyols, amino acids, cyclodextrins, and fructose through fermentative processes.

Since starch is the major storage form of photosynthate of many crops, enhancement of its biosynthesis can lead to an increase of the overall yield of crops. Many important functional properties of the foods and other processed materials derived from starch are affected by the structure and composition of starch, e.g., starch granule shape, amylose-to-amylopectin ratio, crystallinity, phosphorylation, and lipid content, etc. Therefore, there is great interest in increasing the yield and modifying the structure of starch, especially to create novel starches by genetic manipulation of crops. Much effort has been invested in understanding the structure-function relationship of the enzymes directly involved in the starch biosynthetic pathway—ADPGlc PPase, starch synthase, and branching enzyme (1-3, 5-7). Currently, starch is commercially extracted from limited sources, such as maize and potato. Genetic engineering may allow the modification of these starches toward the properties of specialized starches for specific purposes. This review focuses on the studies involving structure-function relationships of ADPGlc PPase, which is a key regulatory enzyme in starch biosynthesis. For discussions on starch synthase and branching enzyme, a number of recent reviews may be referred to (8-11).

2. The Metabolic Path of Carbon to Starch

In actively photosynthesizing leaves, it is well established that the flow of carbon into starch occurs exclusively at the chloroplast (11). The triose phosphate formed in the Benson-Calvin cycle is converted to Glc-1-P via gluconeogenesis to serve as substrate for ADPGlc production, which in turn leads towards the starch synthesis pathway (see below). In young developing leaves that still behave as sink tissues (rather than sources), the carbon source for starch synthesis is believed to be sucrose. In the cytoplasm, sucrose is either hydrolyzed by invertase or by the sequential action of sucrose synthase and UDPGlc PPase (12) to form monosaccharides, which can then enter glycolysis to be converted into triose-phosphates. Triose-P/Pi translocator then transports the triose-phosphates (13, 14) into the chloroplasts where they can be metabolized further for starch synthesis.

In nonphotosynthetic tissues, starch synthesis occurs in the amyloplast. Unlike the autotrophic chloroplasts of leaf tissues, amyloplasts are dependent on the cytoplasm for both energy and carbon. The most accepted pathway for the transport of carbon from the cytoplasm into amyloplast is shown in Figure 1 (15, 16). In the cytoplasm, sucrose is metabolized into hexose-Ps (Glc-6-P and Glc-1-P), which are then transported into the amyloplasts by specific translocators (12, 17-20) for starch synthesis. An alternative pathway of carbon flow into starch in the amyloplasts has been proposed (21). ADPGlc is synthesized directly from sucrose in the cytoplasm and then transported via an ADPGlc translocator into the amyloplast. However, this pathway was believed to play little, if any, role in starch synthesis due to insufficient evidence (16).

3. ADP-glucose Pathway Leads to Biosynthesis of Starch

In the 1950s, the discovery of nucleotide-diphosphate-sugars by Leloir and Cardini (22-24) led to the uncovering of the metabolic routes of polyglucan synthesis. The biosynthesis of starch was first shown by Leloir's group (24, 25) to occur via the

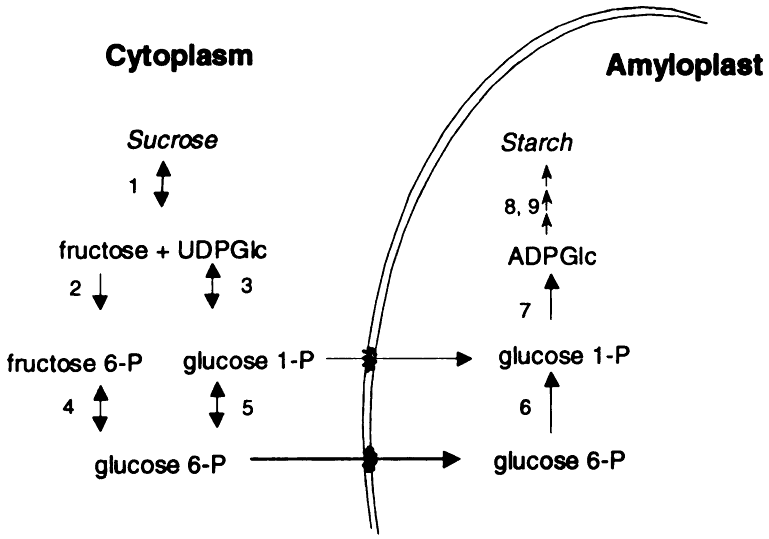

Figure 1. The route leading from carbon into starch in nonphotosynthetic storage organs. In the cytoplasm, sucrose is metabolized into glucose 6-P and glucose 1-P, which are then translocated into the amyloplast via specific translocators (indicated as ovals). Inside the amyloplast, hexose-P is converted into ADPGlc, which in turn enters the starch biosynthesis pathway. The enzymes involved are: 1, sucrose synthase; 2, fructokinase; 3, UDPGlc PPase; 4, cytosolic phosphohexoseisomerase; 5, cytosolic phosphoglucomutase; 6, plastidal phosphoglucomutase; 7, ADPGlc PPase; 8, 9, starch synthase and branching enzyme. This illustration is based on a figure from ref. 10.

transfer of the glucosyl portion of UDP-glucose to an existing starch primer. Subsequently, ADP-glucose was reported as a better glucosyl donor than UDP-glucose (26). Since then, many other groups have confirmed those findings (for reviews, see ref. 8, 11, 27). In most cases, ADP-glucose is the specific glucosyl donor (28).

It is well accepted that the ADP-glucose pathway is the main pathway leading to starch synthesis. This pathway consists of the following three reactions that are required for the synthesis of the α-(1→4) and α-(1→6) glucosidic linkages in amylose and in amylopectin:

1. ATP + α-glucose-1-phosphate $\Leftrightarrow$ ADP-glucose + pyrophosphate

2. ADP-glucose + α-glucan $\Rightarrow$ ADP + α-(1→4)-glucosyl-glucan

3. Linear glucosyl chain of α-glucan $\Rightarrow$ branched chain of α-glucan with α-(1→6) linkage branch points

In the first step, the glucosyl donor, ADP-glucose, is synthesized from ATP and Glc-1-P in a reaction catalyzed by ADPGlc PPase (EC 2.7.7.27). The produced pyrophosphate (PPi) is removed by inorganic pyrophosphatase and this removal makes the reaction irreversible under physiological condition. In the second step, the glucosyl unit of ADP-glucose was transferred to the nonreducing end of a preexisting α-1, 4-glucan chain by starch synthase (EC 2.4.1.21) (24). In the third reaction, branching enzyme (EC 2.4.1.18) catalyzes the formation of α-(1→6) linkages found in amylopectin. The three reactions constitute the so-called ADPGlc pathway of starch synthesis. In bacteria, the synthesis of glycogen shares the same pathway (28).

ADPGlc PPase catalyzes the main regulatory step in this pathway. The supporting biochemical and genetic evidence has been reviewed extensively (8, 11, 15, 16). All the data demonstrated a direct causal relationship between the activity of the ADPGlc PPase and starch accumulation. Some of the most compelling evidence is listed below:

In photosynthetic tissues, two mutants of *Arabidopsis* that were defective in starch synthesis were isolated by Lin et al. (29, 30). One mutant had less than 2% of the wild-type ADPGlc PPase activity resulting in starch accumulation of less than 2% of wild-type level in the leaf. The other mutant was deficient in the large subunit of ADPGlc PPase causing a 93% reduction of the enzyme activity and an accumulation only 26% as much starch in the leaf as compared to the wild-type.

In nonphotosynthetic tissues, ADPGlc PPase was also reported to play a major role in starch synthesis via genetic studies. Two mutants of maize endosperm, *shrunken 2* (*sh2*) and *brittle 2* (*bt2*), which are deficient in ADPGlc PPase activity (5-10% of wild-type), also have deficient levels of starch content (25-30% of wild-type) (31, 32). In potato tuber, the expression of an antisense construct for the small subunit of ADPGlc PPase causes a decrease in enzymatic activity to 2 to 5% of the wild-type level and almost no starch formation (33).

Debranching enzyme has also been suggested to be involved in the formation of amylopectin (34). From this proposal and the information on the various isozymes of branching enzyme and starch synthase, Preiss et al. (6) proposed a pathway for the synthesis of amylopectin and amylose.

4. ADPGlc PPase

The synthesis of ADPGlc by the action of ADPGlc PPase was first reported by Espada in 1962 (35). ADPGlc PPase was later found distributed mainly in the chloroplast and amyloplast of plant tissues (36-38). Nevertheless, two reports proposed that it might also be present in the cytoplasm (39, 40).

4.1. Subunit and Primary Structure

ADPGlc PPase from all sources is tetrameric in structure. The bacterial enzyme is a homotetramer and each subunit has molecular mass between 50 and 55 kDa (28). The plant enzyme consists of two types of subunits: small and large subunits. The small subunits have molecular masses between 50 and 54 kDa, whereas the large subunits have molecular masses between 51 and 60 kDa (8). Interestingly, the cyanobacterial ADPGlc PPase is homotetrameric similar to the bacterial enzyme, but is regulated by 3PGA and Pi like the plant enzyme (41-43).

The structural properties of the spinach leaf enzyme are understood best among the plant ADPGlc PPases (44-46). This enzyme consists of two different subunits with molecular masses of 51 and 54 kDa. Besides the molecular mass, the two subunits are also different in the following properties: N-terminal sequences, amino acid composition, tryptic peptide maps, and antigenic properties (44). Therefore, the two subunits are structurally different and very likely to be the products of two genes.

The potato tuber ADPGlc PPase has been purified to near homogeneity by Okita et al. (47). They observed two different subunits that could be distinguished by their slight differences in the molecular mass (50 and 51 kDa) and in net charge by two-dimensional PAGE. Antibody prepared against the spinach leaf small subunit crossreacts only with the potato tuber small subunit. However, antibody prepared against the spinach leaf large subunit does not react significantly with either subunit of the potato tuber enzyme. The cDNAs of the two subunits were isolated by using antibody against the potato tuber holoenzyme (48-50). The cloned cDNAs were expressed together in an *E. coli* strain devoid of ADPGlc PPase activity (51). Most properties of the recombinant enzyme were similar to the native enzyme purified from potato tuber except that the recombinant enzyme is not heat stable and less sensitive to Pi inhibition. Those differences were linked to the use of an incomplete cDNA for the small subunit, which results in a possible truncation at the N-terminus.

The structural genes for many other plant ADPGlc PPase subunits (small or large) have also been cloned, e.g., those from rice endosperm (49, 52, 53), maize endosperm (54), *Arabidopsis thaliana* (B. Smith-White and J. Preiss, unpublished results), and wheat leaf and endosperm (55). Since 1991, many other genes of plant ADPGlc PPases have been isolated and are not listed here.

Smith-White and Preiss (56) did the first systematic comparison of the primary structures of ADPGlc PPases from different sources. They have found that the small subunit is highly conserved among different plant sources and tissues with an amino acid sequence identity varying from 85 to 95%. The large subunit shares less sequence identity, ranging between 50 and 60%. The identity between the large and small subunits varies between 40 and 60%. Therefore, the conservation of the amino acid sequence of the small subunit from various plant sources is higher than that of the large subunits. On the basis of the identity between the two subunits of the plant ADPGlc PPase and their identity with the homotetrameric bacterial enzyme (~30%), it has been proposed that the two plant subunits may have evolved from the same ancestor (11, 15, 56).

4.2. Regulatory Property

The first report regarding the regulatory property of plant ADPGlc PPase was from Ghosh and Preiss (57). They found that the spinach leaf enzyme was activated by 3PGA and inhibited by Pi. Subsequently, the same regulatory properties were also observed in ADPGlc PPases from other higher plants (11), green algae (15, 58), and cyanobacteria (41, 42). In most cases, 3PGA also increases the affinity for the substrates (ATP and Glc-1-P) and reverses the inhibition by Pi. Based on these findings, it was proposed that the ratio of concentrations for 3PGA and Pi regulates the activity of ADPGlc PPase from plants, which in turn controls the level of starch synthesis (59-62). This ratio fluctuates in response to different physiological conditions during diurnal growth in leaf tissues. For example, CO_2 fixation during photosynthesis in the light leads to the increase of 3PGA concentration, while ATP generation during photophosphorylation leads to the decrease of Pi concentration, indicating that both energy and carbon are in excess. In the dark, the opposite occurs resulting in a decrease of the ratio. Therefore, 3PGA and Pi concentrations within the chloroplast are good indicators of the energy and carbon status. In this regard, the allosteric regulation of ADPGlc PPase provides a good mechanism to control the flux of carbon into starch.

Many studies on both isolated chloroplast systems and intact leaves confirm the existence of this regulation mechanism for plant ADPGlc PPase (for review see ref. 27).

In non-photosynthetic tissues, ADPGlc PPase is also believed to play a dominant role in controlling starch synthesis through the same allosteric regulatory properties as those in photosynthetic tissues. The enzymes derived from potato tuber and maize endosperm are activated by 3PGA 20- to 30-fold (63-64). The enzyme from cassava root is almost completely dependent on 3PGA for the catalytic activity (65). All three enzymes are inhibited by Pi (63-65). However, ADPGlc PPases from pea embryo (66), barley endosperm (67), and bean cotyledon (68) show relative insensitivity to 3PGA activation (1.5- to 3- fold). Preiss and Sivak (8) suggested that proteolysis may be one reason behind the allosteric insensitivity found in those enzymes, which was shown to be the case for the maize endosperm enzyme (64). Furthermore, since the 3PGA activation curve for ADPGlc PPase is sigmoidal, even small changes in the concentrations of 3PGA and Pi produce big changes in the synthetic rate of the enzyme (10). Nevertheless, it is still unknown how the availability of carbon and energy for starch synthesis is signaled in non-photosynthetic systems.

Many *in vitro* and *in vivo* experiments suggest that the allosteric regulation of ADPGlc PPase by 3PGA and Pi are physiologically important for starch synthesis (11, 15, 16, 27, 69). Ball et al. (58) provided important *in vivo* evidence. A starch-deficient mutant of *Chlamydomonas reinhardtii* was found to contain a defective ADPGlc PPase that was less responsive to activation by 3PGA. Other supporting evidence was provided by Stark et al. (70). They increased starch content up to 60% in potato and 10-fold in cultured tobacco cells by transformation with a mutant *E. coli* ADPGlc PPase gene. The mutant gene encoded an enzyme that can synthesize glycogen at a rate three times higher than the wild-type enzyme in *E. coli* and was insensitive to the regulatory effectors of the plant enzymes. In contrast, no increase of starch was observed when wild-type *E. coli* ADPGlc PPase gene was used.

Neuhaus and Stitt (62) used a mutant *Arabidopsis thaliana* to measure the flux control coefficient for ADPGlc PPase by the Kacser-Burns control analysis method (71, 72). In this method, the relationship between the rate of a metabolic process and the activity of an enzyme of interest was calculated to determine the relative importance of that enzyme in regulating the metabolic pathway. It was found that ADPGlc PPase exercises the most significant effect in controlling starch synthesis compared with plastidal phosphoglucomutase and phosphoglucoisomerase.

A light-mediated activation mechanism has been proposed by Kaiser and Bassham (73). They observed that high concentration of DTT could stimulate by 5.7-fold the ADPGlc synthesis in the crude extract of spinach chloroplast. However, neither the crude extract nor the purified ADPGlc PPase could be activated by DTT or by various spinach leaf thioredoxins (27). Furthermore, no light activation could be observed on ADPGlc PPase from intact chloroplasts of either the pea or the spinach (L. E. Anderson, T. Ashton, R. Scheibe, and J. Preiss, unpublished results). Since ADPGlc synthesis was measured from Glc-6-P, it is possible that the phosphoglucomutase activity, which is needed to convert Glc-6-P to Glc-1-P, was actually activated by DTT resulting in the activation of ADPGlc synthesis (27). Nevertheless, ADPGlc PPase purified from potato tuber has been shown to be activated by DTT (63, 74). However, the mechanism of DTT activation was not clear.

4.3. Requirement of Two Subunits for Plant ADPGlc PPase

Many experiments indicate that both the small and large subunits are required for optimal activity of the plant enzyme. As already mentioned, the *bt 2* and *sh 2* mutations in maize endosperm reduce 90 to 95% of ADPGlc PPase activity, causing up to 75% reduction of starch (31, 32, 75, 76). Immunoblotting experiments showed that the *bt 2* and *sh 2* mutants lacked the small and large subunits, respectively (11, 76, 77). In pea, mutation of the *rb* locus affects a gene encoding the large subunit causing 90% or greater reduction of ADPGlc PPase activity and a concomitant up to 62% drop in starch accumulation (66, 78). Li and Preiss (79) have reported that mutation of the large subunit in *Arabidopsis* resulted in an ADPGlc PPase that has activity only in the presence of high concentrations of the activator. Other studies have also showed that the absence of one subunit results in very low ADPGlc PPase activity (29, 31, 51, 80).

Multiple genes were reported to encode the large subunits of many plant ADPGlc PPases, e.g., potato (48), barley (81, 82), *Arabidopsis* (83), rice (52), and wheat (55). The expression of those genes show strong source-specificity, i.e. being restricted to different plant tissues (e.g., leaf, stem, guard cells, tuber, endosperm, root) (55, 56, 81, 82). Thus, it is possible that the different large subunits confer different regulatory properties for the heterotetrameric ADPGlc PPases from different plant species and/or tissues. Further discussion about the function of the two subunits is presented in Chapter 2.

4.4. Substrate Sites

Substrates were shown to bind ADPGlc PPases in an ordered mechanism—ATP binds first, followed by the binding of Glc-1-P (84-86). Product release is ordered with PPi released first, followed by ADPGlc (84). Many studies on the substrate and regulatory site of ADPGlc PPase were started with the bacterial enzyme (87-93). These studies prove to be quite relevant for later studies on the plant counterpart because specific regions, e.g., the substrate and regulator sites, are highly conserved in bacterial and plant enzymes.

Studies to target the substrate sites were mainly pursued by the methods of chemical modification (by using substrate analogs) and site-directed mutagenesis. For example, Pyridoxal 5′-P (PLP), a reagent for modification of lysine residues of proteins, has been used successfully with the *E. coli* ADPGlc PPase. Modification of *E. coli* ADPGlc PPase with [^{3}H]PLP resulted in labeling of two lysine residues, Lys^{39} and Lys^{195} (91, 92). Lys^{39} was shown later to be located in the activator site (91). Modification of Lys^{195} resulted in a loss of catalytic activity that could be prevented by inclusion of ADPGlc and $MgCl_2$. Therefore, Lys^{195} is required for either substrate binding or for catalytic activity. Further study by site-directed mutagenesis showed that Lys^{195} was specifically involved in the binding of one substrate, Glc-1-P, but has no role in the rate-determination step (88). Both the charge and size of this lysine are important for proper binding according to kinetic studies on a series of Lys^{195} mutants (88). Figure 2 shows the alignment of the amino acid sequences of the Lys^{195} region in the *E. coli* ADPGlc PPase with sequences of ADPGlc PPases from other sources. This lysine residue and its surrounding sequences are highly conserved in the bacterial enzymes as well as in both the small and large subunits of plant ADPGlc PPases, suggesting they may perform the same function. Chapter 3 presents the study on the conserved lysine residues in the small and large subunits of ADPGlc PPase of potato tuber.

Tyr^{114} has been identified as being involved in the binding of the other substrate, ATP, in *E. coli* ADPGlc PPase (87, 93). It is located in a region predicted to form a Rossman-fold supersecondary structure, which is a common nucleotide-binding motif in proteins (94). This region is highly conserved in all ADPGlc PPases sequenced to date (8) except that tyrosine is substituted by phenylalanine in the cyanobacterial and plant enzymes. On this basis, it was proposed that those conserved sequences were also involved in the binding of ATP. However, recent site-directed mutagenesis experiment indicated that the equivalent phenylanaline in the cyanobacterial enzyme was not involved in the binding of ATP (J. Sheng and J. Preiss, unpublished results). Therefore, further experiments are necessary to clarify the role of this conserved region. One possibility is that other residues in this region are actually involved in the binding of ATP in cyanobacterial and plant enzymes.

4.5. Regulatory Sites

Due to the importance of the regulatory properties of ADPGlc PPase to starch synthesis, much effort has been directed toward understanding the structural basis for the allosteric regulation of this enzyme (11, 15, 95). The first structural study on the regulatory site of plant ADPGlc PPase was performed on the enzyme from spinach leaf.

Prokaryotes	195
E. coli	IIEFVEKP-AN
S. typhimurium	**D*****-**
Anabaena	V*D*S***KGE
Synechocystis	*TD*S***QGE
Small subunit of the plant enzyme	198
Potato tuber (50 kDa)	****A***QGE
Spinach leaf (51 kDa)	****A***KGE
Rice seed	*V**A***KGE
Maize endosperm (54 kDa)	****A***KGE
Arabidopsis thaliana	****A***KGE
Wheat endosperm	****A***KGE
Large subunit of the plant enzyme	213
Potato tuber (51 kDa)	VVQ*A***KGF
Spinach leaf (54 kDa)	VLS*S***KGD
Maize endosperm (60 kDa)	VLQ*F***KGA
Wheat endosperm	VVQ*S*Q*KGD

Figure 2. Alignment of the amino acid sequences around the Glc-1-P site (Lys^{195}) of ADPGlc PPase from *E. coli* with those from other sources (after Preiss and Sivak, ref. 8). The numbers on top of all the sequences correspond to Lys-195 of the *E. coli* enzyme. The positions of the equivalent lysine residues in the small and large subunit of potato enzyme are also indicated. The conserved residues are underlined in all the sequences. *signifies the same amino acid as in the *E. coli* enzyme.

By using an activator analogue, PLP, four lysine residues [one on the small subunit (45) and three on the large subunit (46, 96)] were found to be potential activator sites (Table I). The activator (3PGA) prevented the modification of all those lysine residues, while the inhibitor (Pi) prevented the modification of two lysine residues: site-1 lysine of the small subunit and site-2 lysine of the large subunit. It is believed that site 1 and site 2 play the most important role in the binding of the activator (8).

The sequence alignment shows that there is high conservation of both site 1 and site 2 regions in the cyanobacterial enzyme and the two subunits of plant enzymes, though the site 1 region shares higher homology (Table I). Studies on the conserved residues (Lys^{419}-site 1; Lys^{382}-site 2) in the homotetrameric *Anabaena* enzyme confirmed that these residues were involved in the binding of the activator (89, 90). Interestingly, recent mutagenesis studies indicate that Lys^{404} (site 2) and Lys^{441} (site 1) on the small subunit of the potato tuber enzyme are more important for the binding of 3PGA than their counterparts (Lys^{417}-site 1 and Lys^{455}-site 2) in the large subunit (97). Substitution of Lys^{404} and Lys^{441} on the small subunit to alanine decreased the apparent affinity for 3PGA 3090- and 54-fold, respectively. Furthermore, the apparent affinity for the inhibitor (Pi) was also affected by the two substitutions, resulting in a more than 400-fold decrease. In contrast, when both Lys^{417} (site 2) and Lys^{455} (site 1) on the large subunit were mutated to alanine, the apparent affinity for the activator decreased only 9- and 3-fold, respectively. These mutations gave negligible effects on the apparent affinity for Pi. It appears that both site 1 and site 2 of the small subunit are important for the binding of the activator as well as the inhibitor, but their counterparts on the large subunit are relatively unimportant.

Regarding inhibitor (Pi) site, Arg^{294} of the *Anabaena* enzyme was shown to be specifically involved in the binding of Pi (98). This residue is conserved in the ADPGlc PPases from cyanobacteria and plants, but not in the enteric bacterial enzymes. Both the

Table I

Sequence Alignment of the Regulatory Sites of ADPGlc PPases

From Plants and Cyanobacteria

(*) signifies that the sequence has been determined by amino acid sequencing (43). (**) signifies that the same sequence in this region has been reported by Park and Chung, accession number, u85496. After Ballicora, et al., ref. 97.

Table I

Accession Number	Source	Tissue	Site	Site
	Cyanobacterial		**Site 2**	**Site 1**
			382	419
Z11539	*Anabaena*	-	DTIIRRAIID**K**NARIG	IVVVL**K**NAVITDGTII
M83556	*Synochocystis*	-	GTTIRRAIID**K**NARIG	IVVVI**K**NVTIADGTVI
	Small subunits			
			404	441
	Solanum tuberosum	Tuber	NCHIKRAIID**K**NARIG	IVTVI**K**DALIPSGIII
x83500	*Spinacia oleracea*	Leaf	NSHIKRAIID**K**NARIG	IVTVI**K**DALIPSGTVI
L33648	*Solanum tuberosum, R.Burbank*		NCHIKRAIID**K**NARIG	IVTVI**K**DALIPSGIVI
x61186	*Solanum tuberosum, R.Burbank*	Tuber	NCHIKRAIID**K**NARIG	IVTVI**K**DALIPSGIII
x55155	*Solanum tuberosum, Desiree*	Tuber	NCHIKRAIID**K**NARIG	IVTVI**K**DALIPSGIVI
x55650	*Solanum tuberosum, Desiree*	Tuber	NCHIKRAIID**K**NARIG	IVTVI**K**DALIPSGIVI
x91736	*Chlamydomonas reinhardti*	-	NSVITNAIID**K**NARVG	ILVID**K**DALVPTGTTI
x76941	*Vicia faba (VfAGPP)*	Cotyledons	NSHIRRAIID**K**NARIG	IVTVI**K**DALIPSGTVI
x76940	*Vicia faba (VfAGPC)*	Cotyledons	NSHIKRAIVD**K**NARIG	IVTII**K**DALIPSGTVL
x96764	*Pisum Sativum*	Cotyledons	NSHIKRAIID**K**NARIG	IVTVI**K**DALIPSGTVI
x96765	*Pisum Sativum*	Cotyledons	NSHIKRAIVD**K**NARIG	IVTII**K**DALIPSGTVI
z79635	*Ipomoea batatas (psTL1)*	Tuberous Root/Leaf	NSHIKRAIID**K**NARIG	IVTII**K**DALIPSGTII
Z79636	*Ipomoea batatas (psTL2)*	Tuberous Root/Leaf	NSHIKRAIID**K**NARIG	IVTVI**K**DALIPSGTVI
L41126	*Lycopersicon esculentum*	Fruit	NCLYKRAIID**K**NARIG	IVTVI**K**DALIPSGIVI
J04960	*Oryza sativa*	Endosperm	NCHIRRAIID**K**NARIG	IVTVI**K**DALLLAEQLYE
M31616	*Oryza sativa*	Leaf	NCHIRRAIID**K**NARIG	IVTVI**K**DALLLAEQLYE
X66080	*Triticum aestivum*	Leaf	NSHIKRAIID**K**NARIG	IVTVI**K**DALLPSGTVI
Z48562	*Hordeum Vulgaris*	Starchy endosperm	NSHIKRAIID**K**NARIG	IVTVI**K**DALLPSGTVI
Z48563	*Hordeum Vulgaris*	Leaf	NSHIKRAIID**K**NARIG	IVTVI**K**DALLPSGTVI
	Zea Mays, (brittle-2)	Endosperm	NSCIRRAIID**K**NARIG	IVTVI**K**DALLPSGTVI
S72425	*Zea Mays*	Leaf	NSHIRKAIID**K**NARIG	IVTVI**K**DALLPSGTVI
	Large Subunits			
			417	455
	Solanum tuberosum	Tuber	NTKIRKCIID**K**NAKIG	IIIIL**EK**ATIRDGTVI
-	*Spinacia oleracea (*)*	Leaf	...IKDAIID**K**NAR..	ITVIF**K**NATIKDGVV
x76136	*Solanum tuberosum, Desiree*	Tuber	NTRIKDCIID**K**NARIG	ITVIS**K**NSTIPDGTVI
x61187	*Solanum tuberosum, R.Burbank*	Tuber	NTKIRKCIID**K**NAKIG	IIIIL**EK**ATIRDGTVI
x74982	*Solanum tuberosum, Desiree*	Leaf	NTKIQNCIID**K**NAKIG	ITVIM**K**NATIKDGTVI
u81033	*Lycopersicon esculentum*		NTKIRKCIID**K**NAKIG	IIIIS**EK**ATIRDGTVI
u88089	*Lycopersicon esculentum*		NTKIRKCIID**K**NAKIG	IIIIA**EK**ATIRDGTVI
U81034	*Lycopersicon esculentum (**)*		NTKIQKCIID**K**NAKIG	ITVIM**K**NATIKDGTVI
x96766	*Pisum Sativum*	Cotyledons	NTKIKNCIID**K**NAKIG	ITIIM**EK**ATIEDGTVI
x78900	*Beta vulgaris, Zuchtlinie*	Tap root	NTKIKNCIID**K**NAKIG	ITIIL**K**NATIQDGLVI
x14348	*Triticum aestivum, cv. Mardler*	Leaf	NTSIQNCIID**K**NARIG	ITVVL**K**NSVIADGLVI
x14349	*Triticum aestivum*	Endosperm	NTKISNCIIDMNARIG	IVVIQ**K**NATIKDGTVV
z21969	*Triticum aestivum*	Dev. Grain	NTKISNCIIDMNARIG	IVVIQ**K**NATIKDGTVV
x14350	*Triticum aestivum*	Endosperm	NTKISNCIIDMNARIG	IVVIQ**K**NATIKDGTVV
z38111	*Zea Mays*	Embryo	NTKISNCIIDMNCQGW	IVVVL**K**NATIKDGTVI
s48563	*Zea Mays, (Shrunken-2)*	Endosperm	NTKIRNCIIDMNARIG	IVVIL**K**NATINECLVI
u66041	*Oryza sativa*	Endosperm	NTKIRNCIIDMNARIG	IVVIL**K**NATNATIKHGTVI
u66876	*Hordeum vulgaris*	Leaf	NTSIQNCIID**K**NARIG	ITVVL**K**NSVIADGLVI
x67151	*Hordeum vulgaris*	Starchy endosperm	NTKISNCIIDMNARIG	IVVIQ**K**NATIKDGTVV

cyanobacterial and plant enzymes are inhibited by Pi but not by AMP, an inhibitor of the bacterial enzymes. In the spinach leaf enzyme, one or more arginine residues were suggested to be involved in the allosteric regulation. However, the specific residue(s) has not been identified (99).

Besides the approach of chemical modification and site-directed mutagenesis, random mutagenesis has also been used to target the regulator sites in the large subunit of potato tuber enzyme. In one study, Pro^{55} (52 in ref. 100) was replaced by leucine and resulted in a mutant enzyme with 45-fold lower affinity for 3PGA (100). However, since the mutation occurred in a loop region, the effect could be caused by a conformational change. In another study, Asp^{416} (413 in ref. 101) was mutated to alanine resulting in a mutant enzyme whose affinity for 3PGA decreased about 6- to 10- fold. This effect is similar to that observed when a neighboring residue, Lys^{417}, was mutated to alanine (97). The moderate effects of the mutations on these two residues suggest that they may not be critical for the binding of the activator.

An *in vivo*, transposon mediated mutagenesis system was used by Giroux et al. (103) to introduce short insertions to the C-terminal region of *sh 2,* a gene encoding the large subunit of ADPGlc PPase in the maize endosperm. A mutant line was isolated with an 11-18% increase of seed weight. Further analysis showed that the starch content and several seed components were increased. The mutant ADPGlc PPase was less sensitive to Pi inhibition, but with normal response to 3PGA activation. Sequence analysis revealed that two amino acids (tyrosine and serine) were inserted at the N-terminal side (about 10 amino acids) of the site-1 lysine. Insertion of tyrosine and serine at the corresponding position of the potato large subunit resulted in a heterotetrameric recombinant enzyme that is insensitive to Pi inhibition. Therefore, the insertion region on the large subunit comprises part of the allosteric domain.

4.6 Secondary Structure of ADP-Glucose Pyrophosphorylase

As mentioned in the preceding text, various mutagenesis and chemical modification methods have provided much useful information on the structure-function relationship of the substrate and activator sites of ADPGlc PPases. However, a three-dimensional structure is needed so that we can fit all those "jigsaw pieces" together to thoroughly uncover the catalytic and regulatory mechanism of this enzyme. The *E. coli* enzyme has been crystallized (104) but the crystals are of poor diffraction quality and sensitive to x-ray exposure.

Despite lack of knowledge regarding the three-dimensional structure, a general secondary structure of ADPGlc PPases was predicted by Ballicora et al. (102). In the first step, a hydrophobic cluster analysis method (105) was applied to ADPGlc PPases from *E. coli*, *Anabaena,* and potato tuber. It was found that all of these enzymes share identical hydrophobic clusters in many regions and are different only in some insertions/deletions. The insertions/deletions contain many hydrophilic amino acids, suggesting they are not part of the "core" structure of the protein. These observations indicate that ADPGlc PPases share a common folding pattern in spite of their different quaternary structures (homotetrameric in bacteria and heterotetrameric in plants) and different specificity for the regulators.

In the second step, the amino acid sequences of *E. coli* and *Anabaena* ADPGlc PPases together with the two subunits of potato tuber enzyme were analyzed by the PHD program (106), and a general secondary structure was obtained (Figure 3). The enzyme has five major domains. Domains 1, 2 and 4 consist of α/β structure while domain 3 and 5 mainly consist of β sheets. Good agreement was observed between this model and biochemical data, especially those from the limited proteolysis studies with the *E. coli* (M. -X. Wu and J. Preiss, manuscript submitted) and *Anabaena* enzymes (Y. -Y. Charng and J. Preiss, unpublished results; J. Sheng and J. Preiss, unpublished results). All the

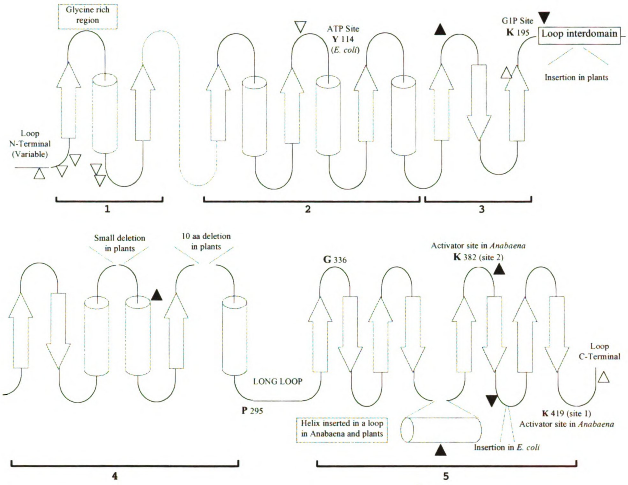

Figure 3. Pediction of the secondary structure of ADP-glucose pyrophosphorylase (after Ballicora, et al., ref. 102). Triangles indicate protease cleavage sites. The topology between regions 1 and 2 cannot be ascertained and is drawn as a dotted line.

protease digestions occur at the predicted loop regions with one exception, which lies in an α helix insertion in domain 5 of the *Anabaena* and plant enzymes. However, since this helix is predicted to be exposed, it is most likely to be a part of a loop region. Most of the conserved residues that are important for the binding of substrates (Tyr^{114} and Lys^{195} in *E. coli*) and activators (Lys^{39} in *E.* coli; Lys^{419} and Lys^{382} in *Anabaena* representing sites 1 and 2, respectively) are located in loops, or very close to loops. Pro^{195} and Gly^{336} of the *E. coli* enzyme that are important for the regulatory properties (107, 108) are also located in the loop regions.

Domain 2 contains a Rossmann fold supersecondary structure, in which the ATP binding site of *E. coli* enzyme is located. It is suggested that domains 1, 2, and 3 form a catalytic core with substrates binding on the top (according to the orientation depicted in Figure 3).

Recently, ADPGlc PPase from *E. coli* was treated with proteinase K to characterize different structure domains (M. -X. Wu and J. Preiss, manuscript submitted). The cleavage sites close to the Glc-1-P binding site (Lys^{195} in *E. coli* enzyme) are protected by ADP-glucose, Mg^{2+}, and FBP (activator). When the *E. coli* enzyme was treated with proteinase K in the presence of ADP-glucose, Mg^{2+}, and FBP, 11-13 amino acids from the N-terminus and 2 amino acids from the C-terminus were cleaved. The truncated enzyme retained maximal activity, but was no longer sensitive to inhibition and did not require the activator for increase in activity. Thus, the N-terminal loop was suggested to be involved in the allosteric regulation of the *E. coli* enzyme.

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CHAPTER 2

THE SMALL SUBUNIT OF POTATO TUBER ADP-GLUCOSE PYROPHOSPHORYLASE HAS CATALYTIC ACTIVITY

Part of the data were published in the issue of *Plant Physiol.* (1995) **109**, 245-251 by Ballicora, M. A., Laughlin, M. J., Fu, Y., Okita, T. W., Barry, G. F., and Preiss, J.

Abstract

The different properties between a recombinant and native ADP-glucose pyrophosphorylase of potato tuber were ascribed to a possible truncation at the N-terminus of the small subunit based on the conservation of the N-terminal sequence between the potato tuber and spinach leaf small subunit. In this work, the cDNA of the truncated small subunit was extended by ten amino acids at its N-terminus. Significantly, expression of this extended cDNA of the small subunit in *E. coli* yielded a catalytically active enzyme. After purifying to homogeneity, its specific activity (assayed at high concentration of activator) is similar to that of the purified heterotetrameric enzyme containing both the small and large subunits. The activity of the large subunit alone is negligible. The homotetrameric small subunit differs from the heterotetrameric enzyme only in its regulatory properties. Therefore, the small subunit is proposed to be mainly involved in catalysis, while the large subunit is mainly involved in modulating the regulatory properties of the small subunit of plant enzymes. The properties of the heterotetrameric enzyme containing the large and extended small subunits are more similar to those of the native enzyme than the heterotetrameric enzyme, which contained the large and truncated small subunits. The N-terminus of the small subunit may be involved in establishing the regulatory properties and conferring the heat stability of the holoenzyme.

Introduction

In spite of the importance of starch synthesis in reserve tissues, e.g., potato tuber, no study has been done on the protein structure of the potato tuber enzyme due to the difficulty in obtaining large amounts of pure enzyme. The genes of both the small and large subunits of the potato tuber enzyme have been cloned (1). The cloned cDNAs have been expressed together using two compatible vectors in an *E. coli* mutant devoid of ADPGlc PPase activity (2). The recombinant enzyme is similar to the native enzyme purified from potato with respect to most properties. However, two major differences exist between them: the recombinant enzyme exhibits low heat stability and less sensitivity to Pi inhibition (2). ADPGlc PPases from different sources have been reported to be stable at 60-70 °C for 5 min (3-5). Heat treatment was used as a purification step for the native enzyme from potato tuber (3), but it was not possible to use it as a purification step for the recombinant enzyme (2). By comparison with the mature small subunit from spinach leaf, it was found that the cloned small subunit from potato tuber lacked 10 amino acids of the N-terminus (Table I). The spinach leaf enzyme is the only ADPGlc PPase whose small subunit N-terminal sequence has been determined by protein sequencing (4). Previous attempts to sequence the N-terminus of the subunits of the native potato tuber enzyme were unsuccessful. The overall amino acid sequence of the potato tuber small subunit shows 95% identity to that of the spinach leaf small subunit and their N-terminal sequences are almost the same (1, 6). It was proposed that the reason to account for the different properties between the recombinant and native enzyme was due to the truncation of about 10 amino acids at the N terminus of the potato tuber small subunit (2).

In this work another plasmid was constructed that contains a cDNA encoding the small subunit of the potato tuber enzyme with 10 more amino acids at the N-terminus. The extended polypeptide was named small subunit to distinguish from the truncated

Table I

N-terminal Amino Acid Sequences of the Small Subunits of ADPGlc PPases from Potato Tuber and Spinach Leaf[a]

source of small subunit	amino acid sequence
potato tuber cDNA clone	^{66}MIVSPKAVSDSQNSQTCLDPDA...
pMON17335	MALDPDA...
pML10	MAVSDSQNSQTCLDPDA...
spinach leaf	VSDSQNSQTCLDPEA...

[a] The amino acid sequence of the small subunit from potato tuber cDNA clone (1) was deduced from nucleotide sequence, so were those encoded by pMON17335 (2) and pML10. The N-terminal sequence of the small subunit encoded by pML10 was further verified by protein sequence as described in "Materials and Methods". The sequence of the small subunit from spinach leaf was determined by protein sequencing (4). The position number of the methionine residue in the small subunit from potato tuber cDNA clone is according to Nakata et al. (1). The underlined amino acids are those added to the N-terminus of the truncated small subunit.

small subunit used in the previous study. Significantly, the expression of this extended small subunit yielded a homotetrameric enzyme with catalytic activity comparable to the heterotetrameric holoenzyme when determined in the presence of high concentrations of 3PGA, whereas the large subunit had negligible catalytic activity. The possible roles of the small and large subunits of the plant ADPGlc PPases are discussed. The recombinant enzyme that contained the large and extended small subunits is heat stable and is as sensitive to Pi inhibition as the native enzyme purified from potato tuber.

Materials and Methods

Reagents

ATP, ADPGlc, Glc-1-P, and 3PGA were purchased from Sigma. [^{14}C]Glc-1-P and ^{32}PPi were purchased from DuPont-New England Nuclear. All other reagents were of the highest available commercial grade.

Plasmids

The construction of plasmids pMON17335 and pMON17336, which contain cDNAs encoding the truncated small subunit and large subunit of ADPGlc PPase of potato tuber, respectively, was described before (2). For generation of the extended cDNA for the small subunit, a methionine was engineered at position 71 (Table I) of the cDNA clone of the potato tuber small subunit to introduce an *Nco*I site using the following oligonucleotide: 5′-GGGGTCGCCCATGGCTGTTTCTGATTCG-3′. After amplification by PCR, the *Nco*I/*Kpn*I fragment was ligated to pMON17335 digested with *Nco*I/*Kpn*I to form pML10. The entire coding region was verified by sequencing. The newly generated cDNA encoding a polypeptide with 10 more amino acids at the N-terminus than the truncated small subunit encoded by the cDNA in pMON17335 (Table I).

Assay of ADPGlc PPase

(A) Assay I. In the pyrophosphorolysis direction, enzyme activity was assayed according to a previously described method (4). The reaction mixture contained 80 mM glycylglycine, pH 8.0, 2 mM ADPGlc, 5 mM $MgCl_2$, 3 mM DTT, 1.5 mM ^{32}PPi (1,000-2,000 cpm/nmol), 3 mM 3PGA, 10 mM NaF, 200 μg mL^{-1} of BSA, and enzyme in a total volume of 250 μL.

(B) Assay II. In the ADPGlc synthesis direction, enzyme activity was measured according to a previously described method (7). The reaction mixture contained 100 mM Hepes-NaOH, pH 8.0, 0.5 mM [^{14}C]Glc-1-P (1,000-3000 cpm/nmol), 1.5 mM ATP, 5 mM $MgCl_2$, 3 mM DTT, 200 μg mL^{-1} of BSA, 0.3 unit of inorganic pyrophosphatase, and

enzyme in a final volume of 200 μL. In the activated condition, the concentration of 3PGA used in *Assay II* was 3 mM for the heterotetrameric enzyme and 24 mM for the homotetrameric small subunit.

Kinetic Studies

For determination of kinetic parameters, the concentration of the substrate or effector tested was systematically varied with the other substrates and effectors fixed at a saturating concentration as described in *Assay I* or *II.* Kinetic data were plotted as initial velocity *versus* substrate or effector concentration. The kinetic constants, $A_{0.5}$, $S_{0.5}$, and $I_{0.5}$, which correspond to the concentration of activator, substrate, and inhibitor giving 50% of maximal activation, velocity, and inhibition, respectively, were obtained from a computer program using nonlinear iterative least-squares fitting to a modified Michaelis-Menten equation (8).

Protein Assay

Protein concentration was measured using the Pierce bicinchoninic acid (BCA) reagent (9) with BSA as the standard.

Molecular Mass Determination

The molecular weights of the recombinant heterotetrameric enzyme and the homotetrameric small subunit were determined on a 5 to 20% sucrose density gradient in 50 mM Hepes, pH 7.5, 2 mM EDTA (10). The purified small subunit and the recombinant enzyme containing both the extended small and large subunits had essentially the same molecular masses of 201 ± 13 and 202 ± 20 kDa, respectively. Since the large and small subunits have molecular masses of 51 kDa and 50 kDa, respectively, both enzymes have tetrameric structure. The heterotetrameric enzyme was shown to have two large subunits and two small subunits (11).

Expression in *E. coli*

Competent cells of *E. coli* mutant strain AC70R1-504 were transformed either with two compatible plasmids (pML10 or pMON17335 with pMON17336) or with one plasmid (pML10, pMON17335, or pMON17336). The transformed bacteria were grown in LB medium containing 25 μg/mL kanamycin and/or 70 μg/mL spectinomycin at 37 °C on a rotary shaker. Cells were grown until OD_{600}=1.2-1.4 and then transferred to room temperature to add 500 μM IPTG and/or 5 μg/mL nalidixic acid for induction (Table II).

Table II

Expression Conditions of Plasmids Containing the cDNAs encoding the Subunits of ADPGlc PPase of Potato Tuber

plasmid	subunit	antibiotics	inducer
pML10	small subunit	kanamycin	IPTG
pMON17335	truncated small subunit	kanamycin	IPTG
pMON17336	large subunit	spectinomycin	nalidixic acid

After ~ 40h of expression, cells were harvested at 3, 000 g for 15 min at 4 °C. The pellet was resuspended in buffer A [50 mM Hepes-NaOH, pH 8.0, 5 mM $MgCl_2$, 1 mM EDTA, and 20% (w/v) sucrose] at a ratio of 5 mL buffer/g of cell. Cells were then disrupted by sonication in a Heat Systems Ultrasonic Sonicator model W-220F and then centrifuged at 12,000 g for 15 min. The supernatant was immediately subjected to a heat treatment procedure as indicated below. With this improved procedure for expression, the specific activity of the recombinant enzyme (large + small) in the crude extract was at least 20 times higher (1.4-2.0 units/mg) than that reported before (2).

Heat Treatment

Crude extract (0.2 mL) was heated for 5 min at the indicated temperature in a 1.5-mL microcentrifuge tube and then placed immediately in ice water. The treated sample was centrifuged at 14,000 g for 10 min at 4 °C to collect supernatant. Aliquots were withdrawn from the supernatant to assay activity in the pyrophosphorolysis direction (*Assay I*). The sample without treatment was used as the control. When used as a purification step, the crude extract was heated in a water bath at 65°C for the heterotetrameric enzyme (small + large subunits) and 70 °C for the small subunit in an Erlenmeyer flask with continuous agitation. The sample was kept for 5 min when the desired temperature was reached, then immediately placed in an ice-water bath. The supernatant was collected by a centrifugation step at 15,000 g for 15 min before being frozen in liquid nitrogen. The heat-treated sample was kept at -80°C until further purification steps.

Enzyme Purification

The recombinant enzyme containing the large subunits and the truncated small subunits was purified as previously described (2). The small subunit and the recombinant enzyme containing both the small and the large subunits were purified following a described procedure with some modifications (3). The buffers did not contain glutathione and the enzymes were loaded onto the column in the presence of 1.2 M ammonium sulfate instead of 1.3 M potassium phosphate. For purification of the enzyme with both the small and large subunits, an ammonium sulfate precipitation step (50% saturation) was added after the heat treatment step. Then the pellet was dissolved in a minimal volume of buffer A and dialyzed against the same buffer overnight. The dialyzed enzyme was centrifuged at 20,000 g for 15 min at 4°C to remove insoluble materials before being loaded onto the DEAE Fractogel column. The heat treatment step was used for the purification of the small subunit and recombinant enzyme containing both the small and the large subunits, but was not used for the recombinant enzyme containing the large subunit and the truncated small subunit. *Assay I* was used to follow the activity through the purification steps.

SDS-PAGE and Immunoblot Blot

SDS-PAGE was performed according to Laemmli (12) on 10% polyacrylamide gels. The purified small subunit (8 μg) was subjected to electrophoresis in SDS-PAGE and subsequently blotted to a PVDF membrane (Applied Biosystems Inc.). After staining with Coomassie blue R250, the protein band was cut for sequencing as described previously (13). For immunoblot analysis, proteins were transferred to a nitrocellulose membrane 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 alkaline phosphatase -substrate precipitating reagent (Boehringer Mannheim GmbH).

Sequence Determination

Protein sample was applied to Procise (Applied Biosystems model 494A) automated sequencer for amino acid sequence analysis. The N-terminal sequence for the small subunit was: AVSDSQNSQT(X)L This sequence confirms that the putative mature small subunit contains the 10 extra amino acids (Table I). The first methionine was processed. Residue 11, which should be cysteine, was not identified.

Results

Expression of cDNAs Encoding the Different Subunits of ADPGlc PPase

The expression of the cDNAs of ADPGlc PPase 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. It had been shown that the small subunit of the potato tuber ADPGlc PPase could cross-react significantly with the anti-spinach leaf antibody, while the large subunit only reacts weakly (4). Three enzymes (small subunit, truncated small subunit and the heterotetramer containing both the small and large subunits) were produced at a similar level based on the result of immunoblotting (data not shown). Their apparent sizes were the same at about 50-51 kDa.

In the crude extract, expression of pMON17336 (encoding the large subunit) yielded an enzyme with undetectable ADPGlc PPase activity (Table III). Expression of both pMON17336 and pML10, which encoded both the small and large subunits, yielded an enzyme with maximal activity. Significantly, expression of pML10 (encoding the small subunit) resulted in an enzyme with catalytic activity (1.2 units/mg) comparable to that of the heterotetrameric enzyme with the small and large subunits (1.8 units/mg). In contrast, expression of pMON17335 (encoding the truncated small subunit) gave negligible activity, which was consistent with the previous study (2).

Heat Stability

As indicated in Table IV, the heterotetrameric enzyme containing both the extended small subunit and large subunits is heat stable at both 60 °C (89% recovery) and 65 °C (83% recovery). This is similar to the heat stability of the native enzyme from potato tuber (90% recovery at 60 °C and 95% recovery at 65 °C). In contrast, the heterotetrameric enzyme containing the truncated small subunit and large subunit was not stable at 60°C (24% recovery) and was almost completely inactivated at 65°C as previously reported (2).

Table III

Activity of ADPGlc PPases Containing Different Subunit(s) [a]

subunit composition of the enzyme	enzyme activity (units/mg)
none	<0.0003
truncated small subunit	<0.002
large subunit	<0.002
large + truncated small subunits	0.86
large + small subunits	1.8
small subunit	1.2

[a] The activity was assayed in the pyrophosphorolysis direction (*Assay I*) in the crude extract.

The homotetrameric small subunit had a 93% recovery of activity in a 5-min treatment at 70 °C. Under the same condition, the heterotetrameric enzyme containing the large and extended small subunits lost more than 90% of its activity (data not shown). Therefore, the small subunit is more stable than the heterotetrameric enzyme.

Purification of ADPGlc PPases

As shown in Table V, the small subunit was purified 43-fold with 46% yield, resulting in an enzyme with a specific activity of 51.6 units/mg. The heterotetrameric enzyme containing two subunits was purified 33-fold with a specific activity of 63.5 units/mg (Table VI), which is slightly higher than that of the small subunit. The specific activities of both enzymes were close to that of the highly purified native enzyme from potato tuber (56.9 units/mg) (14).

Both enzymes were purified to apparent homogeneity estimated from 4 μg of protein on SDS-PAGE. The heat treatment step appears to give a better purification for

Table IV

Heat stability of ADPGlc PPases from Different Sources [a]

Enzymes	activity recovery (%)	
	60 °C	65 °C
homotetramer (small subunit)	98	102
heterotetramer (large + small subunits)	89	83
Heterotetramer (large + truncated small subunits)	24	1.4
native enzyme [b]	90	95

[a] Heat treatments were done with crude extracts for 5 min at the indicated temperature. One hundred percent activity corresponds to 0.19, 0.17, and 0.04 unit/mg in the homotetramer (small subunit), heterotetramer (large + small subunits), and heterotetramer (large + truncated small subunits) enzymes, respectively. [b] Data is obtained from ref. 3.

Table V

Purification of the Small Subunit of Potato Tuber ADPGlc PPase [a]

step	volume	protein	total activity	specific activity	purification	yield
	mL	*mg*	*units*	*units/mg*	*-fold*	%
crude extract	100	1530	1810	1.2	1.0	100
heat treatment (70 °C)	85	315	1624	5.2	4.3	90
DEAE-sepharose	24	110	1279	11.6	9.7	71
C_3 column	29	35	1119	32.1	26.8	62
mono Q	33	16	826	51.6	43.0	46

[a] Purification was carried out from 21.9 g of *E. coli* cells transformed with pML10.

Table VI

Purification of the Heterotetrameric ADPGlc PPase (small + large subunits) of Potato Tuber [a]

step	volume	protein	total activity	specific activity	purification	yield
	mL	*mg*	*units*	*units/mg*	*-fold*	*%*
crude extract	176	1566	3044	1.9	1.0	100
heat treatment (65 °C)	140	420	1806	4.3	2.3	59.3
30-50% $(NH_4)_2SO_4$	12	39.6	701	17.7	9.3	23.0
DEAE-sepharose	8.2	18.0	653	36.3	19.1	21.5
C_3 column	11	9.4	352	37.4	19.7	11.6
mono Q	8	5.5	349	63.5	33.3	11.5

[a] Purification was carried out from 20.3 g of *E. coli* cells transformed with pMON17336 and pML10.

the homotetrameric small subunit than the heterotetrameric enzyme since it was performed at a higher temperature (70 °C) for the homotetrameric enzyme than the heterotetramer (65 °C).

Activation by 3PGA

Interestingly, although the specific activity of the purified homotetrameric small subunit was similar to that of the purified heterotetrameric enzyme, their allosteric properties toward 3PGA activation were strikingly different (Figure 1A). Unlike the heterotetrameric enzyme, no activity of the small subunit could be detected in the absence of 3PGA (data not shown). The $A_{0.5}$ for the homotetrameric enzyme was 15 times higher in the synthesis direction (Table VII) and 96 times higher in the pyrophosphorolysis direction (data not shown), indicating that the small subunit has a much lower affinity for the activator than the heterotetrameric enzyme.

When determined in the synthesis direction, the $A_{0.5}$ for the recombinant heterotetrameric enzyme containing the complete small subunit was almost 3 times higher than that of the recombinant heterotetrameric enzyme with the truncated small subunit, resulting in an enzyme more similar to the native enzyme (Table VII).

Inhibition by Pi

The homotetrameric small enzyme was much more sensitive to inhibition by Pi. In the presence of 3.0 mM 3PGA, 0.5 mM Pi completely inhibited the activity of the small subunit but only inhibited 40% of the activity of the heterotetrameric enzyme (Figure 1B). At this concentration of 3PGA, the $I_{0.5}$ value (80 μM) of the small subunit was about 8 times lower than that of the heterotetrameric enzyme (Table VII).

In the presence of 0.25 mM 3PGA, the $I_{0.5}$ value (0.07 mM) of the heterotetrameric enzyme containing the large and extended small subunits was more similar to that of the native enzyme (0.12 mM) than that of the heterotetrameric enzyme (0.68 mM) containing the large and truncated small subunits (Table VII).

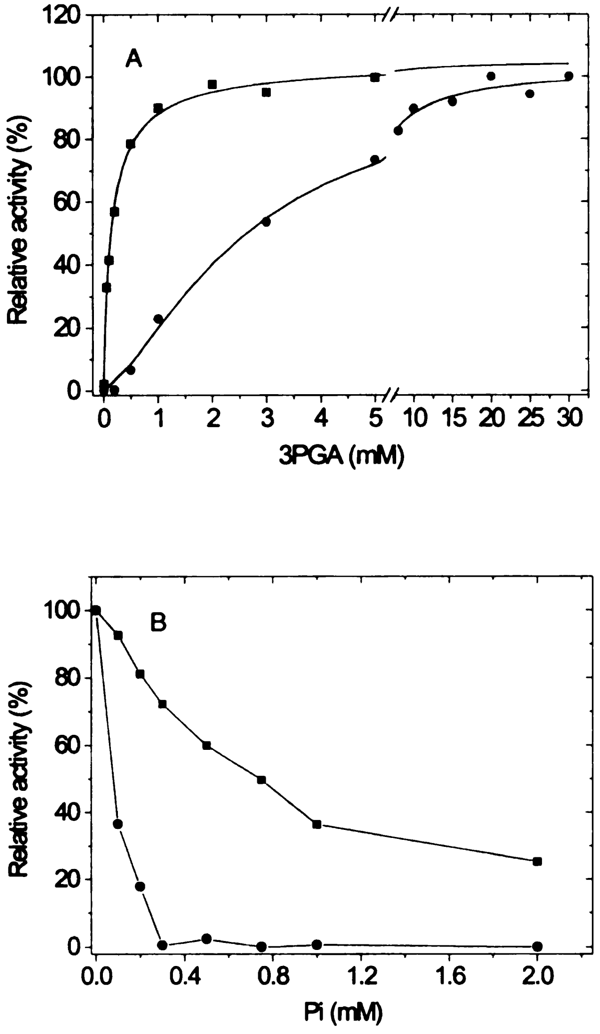

Figure 1. Comparison of the regulatory properties of the homotetrameric small subunit (●) and heterotetrameric enzyme (small + large subunits) (■). Panel A and B show the activation by 3PGA and inhibition by Pi of the two enzymes, respectively. 100% activity corresponds to 6.3 and 5.9 nmol/min in A, 5.8 and 11.3 nmol/min in B, for the homotetramer and heterotetramer, respectively. Assay was determined in the ADPGlc synthesis direction (*Assay II*) as described under Materials and Methods. Pi inhibition was determined in the presence of 3 mM 3PGA.

Table VII

Kinetic Constants for 3PGA and Pi of ADPGlc PPases Purified from Different Sources

Enzymes	$A_{0.5}$ for 3PGA	$I_{0.5}$ for Pi at 0.25 mM 3PGA	$I_{0.5}$ for Pi at 3.0 mM 3PGA
	mM	mM	mM
homotetramer (small subunit)	2.4	--[a]	0.08
heterotetramer (large + small subunits)	0.16	0.07	0.68
heterotetramer (large + truncated small subunits)[b]	0.057	0.68	--
native enzyme[c]	0.4	0.12	0.33

[a] --, not determined. [b] Data is obtained from ref. 2 and has been repeated. [c] Data is obtained from ref. 3. The experiments had been done at least twice and are reproducible to within ±10%.

Kinetic Constants for Substrates

The $S_{0.5}$ values for the substrates Mg^{2+}, ATP, and Glc-1-P were 2.0 mM, 0.120 mM, and 0.040 mM for the heterotetramer and 2.2 mM, 0.200 mM, and 0.029 mM for the homotetrameric small subunit, respectively. No difference of more than 2-fold was observed.

Discussion

The most important observation of this study is that the full-length small subunit of potato tuber ADPGlc PPase is highly active (51.6 units/mg) in the presence of relatively high concentration of 3PGA. Its specific activity is very close to that of the purified native (56.9 units/mg; ref. 14) and cloned (63.5 units/mg) heterotetrameric potato tuber enzyme (14). In contrast, the activity of the large subunit remains undetectable (<0.001 units/mg) even if the concentrations of Mg^{2+}, ADPGlc and 3PGA were raised to 10 mM, 8 mM and 20 mM, respectively. The functional small subunit must exist as a homotetramer since its native form has a molecular mass of 201 kDa. The major differences between the homotetrameric small subunit and the heterotetrameric enzyme are their allosteric regulatory properties. The small subunit requires a much higher concentration of 3PGA for maximal activation and is more sensitive to inhibition by Pi. This is in agreement with a previous study that showed that an *Arabidopsis* mutant ADPGlc PPase lacking the large subunit had activity but with lower affinity for 3PGA (15). The kinetic constants for the substrates are similar between the small subunit and the heterotetrameric enzyme. These observations suggest that: 1. The small subunit is mainly involved in catalysis; 2. The large subunit serves to modulate the allosteric regulation of the small subunit since it increases the affinity for the activator and simultaneously decreases the inhibition by the inhibitor. Regarding the role of the large subunit, Ballicora et al. recently showed that the putative activator sites in the large subunit are not as important as the overall interaction of the large subunit with the small subunit (16). It would be of interest to know if this is a general mechanism for all the plant ADPGlc PPases.

This study provides a basis to answer two important questions: what are the roles of the two subunits in the plant ADPGlc PPases? Why does the plant enzyme have a two-subunit structure *versus* the one-subunit structure of the bacterial counterpart? Since the small subunit is mainly involved in catalysis and all the ADPGlc PPases share the same catalytic mechanism, it becomes apparent that the small subunit is under high selection pressure during evolution (6). The two-subunit structure has the advantage that by altering the large subunit it confers different sensitivity of the small subunit toward allosteric regulation in different plants and different tissues or during different development stages of the same plant, therefore, to allow the plants to accommodate different physiological needs.

By inclusion of 10 amino acids in the N-terminus of the truncated small subunit, a recombinant enzyme was obtained which is more similar to the native enzyme with respect to heat stability and allosteric properties than the recombinant enzyme containing the large and truncated small subunits. The kinetic constants for the substrates were very similar between the recombinant heterotetrameric enzyme, which contains the large and extended small subunit, and the native enzyme (data not shown). Therefore, it can be concluded that the N-terminus of the small subunit is involved in maintaining the native conformation of the protein that is related to the regulatory properties and heat stability. As a matter of fact, chapter 4 shows that an intermolecular disulfide bridge between the N-terminus of the two small subunits is responsible for the heat stability of the heterotetrameric enzyme.

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CHAPTER 3

MUTAGENESIS OF THE GLUCOSE-1-PHOSPHATE BINDING SITE OF POTATO TUBER ADP-GLUCOSE PYROPHOSPHORYLASE

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Abstract

Lys^{195} in the homotetrameric ADPGlc pyrophosphorylase from *Escherichia coli* was shown previously to be involved in the binding of the substrate, glucose-1-phosphate (Glc-1-P). This residue is highly conserved in the ADPGlc pyrophosphorylase family. Site-directed mutagenesis was used to investigate the function of this conserved lysine residue in the large and small subunits of the heterotetrameric potato tuber enzyme. The apparent affinity for Glc-1-P of the wild-type enzyme decreased 135- to 550- fold by changing Lys^{198} of the small subunit to arginine, alanine, or glutamic acid, suggesting that both the charge and size of this residue influence the binding of Glc-1-P. These mutations had little effect on the kinetic constants for the other substrates (ATP and Mg^{2+} or ADP-glucose and PPi), activator (3-phosphoglycerate), inhibitor (Pi), and on the thermal stability. Mutagenesis of the corresponding lysine residue (Lys^{213}) in the large subunit had no effect on the apparent affinity for Glc-1-P by substitution with arginine, alanine or glutamic acid. A double mutant, $S_{K198R}L_{K213R}$, was also obtained with a 100-fold reduction of the apparent affinity for Glc-1-P. The data indicate that Lys^{198} in the small subunit is directly involved in the binding of Glc-1-P, while they appear to exclude a direct role of Lys^{213} in the large subunit in the interaction with this substrate.

Introduction

Chemical modification and site-directed mutagenesis studies have identified that Lys^{195} in ADPGlc PPase from *E. coli* is involved in the binding of the substrate, Glc-1-P (1, 2). This residue is highly conserved in the bacterial enzymes as well as in both the small and large subunits of plant ADPGlc PPases (3). However, no studies have been done to investigate the function of these highly conserved lysine residues in the plant ADPGlc PPases which consist of two different subunits. The expression system for potato tuber ADPGlc PPase (see Chapter 2) provides a useful tool to characterize the role of the corresponding lysine residues in the small subunit, Lys^{198}, as well as in the large subunit, Lys^{213}, and to see if they retain the same function as in the homotetrameric *E. coli* enzyme. As indicated before, recent mutagenesis studies suggest that the small and large subunits can not be simply classified as catalytic and regulatory subunit (4), respectively, this study will also be helpful to further clarify the roles of the small and large subunits.

Materials and Methods

Reagents

ATP, ADPGlc, Glc-1-P, Man-1-P, Gal-1-P, glucuronic acid-1-P, 3PGA, and PPi were purchased from Sigma. ^{32}PPi and [8-^{14}C]ATP were purchased from DuPont NEN. [u-^{14}C]Glc-1-P was from ICN Pharmaceuticals, Inc. The [α-^{35}S]dATP and the *in vitro* mutagenesis kit were from Amersham. Enzymes for DNA manipulation and sequencing were from New England Biolabs and U.S. Biochemical Corp., respectively. Oligonucleotides were synthesized and purified by the Macromolecular Structure Facility at Michigan State University. All other reagents were of the highest available commercial grade.

Bacterial Strains and Media

E. coli strain TG1 [*F'traD36 lacI*^{q} *Δ(lacZ)M15 proA*$^+$*B*$^+$*/supEΔ(hsdM-mcrB)5(r*$_k^-$*m*$_k^-$*McrB*$^-$*) thi Δ(lac-proAB)*] was used for site-directed mutagenesis. *E. coli* mutant strain AC70R1-504 (5), which exhibited negligible ADPGlc PPase activity, was used for expression of the potato tuber ADPGlc PPase gene (6). Both *E. coli* strains were grown in LB medium.

Site-Directed Mutagenesis

For mutagenesis, the gene for the small subunit of potato tuber ADPGlc PPase was subcloned as an *Eco*RI fragment from pML10, a plasmid containing the cDNA gene of the small subunit (6), into the *Eco*RI site of M13mp18RF. The gene for the large subunit was subcloned as an *Xba*I fragment from pMON17336, a plasmid containing the cDNA gene of the large subunit (6, 7), into the *Xba*I site of M13mp19RF. After mutagenesis, in the case of the small subunit, the mutated *Eco*RI fragment was exchanged with the unmutated *Eco*R I fragment in pML10; for the large subunit, the mutated *Xba*I fragment was exchanged with the unmutated *Xba*I fragment in pMON17336. Site-directed mutagenesis experiments were performed according to a previously described method (8) using the *in vitro* site-directed mutagenesis kit from Amersham. Three heterotetrameric mutant enzymes with a single substitution of arginine, alanine, and glutamic acid at Lys^{198} of the small subunit were designated as $S_{K198R}L_{wt}$, $S_{K198A}L_{wt}$, and $S_{K198E}L_{wt}$, respectively. The mutant enzymes with substitution of arginine, alanine, and glutamic acid at Lys^{213} of the large subunit were designated as $S_{wt}L_{K213R}$, $S_{wt}L_{K213A}$, and $S_{wt}L_{K213E}$, respectively. The double mutant enzyme, in which both Lys^{198} of the small subunit and Lys^{213} of the large subunit were replaced with arginine, was designated as $S_{K198R}L_{K213R}$. The oligonucleotides used to create those desired mutations are shown in Figure 1. Prior to expression of the genes for mutant enzymes, the entire coding regions of these mutant alleles were sequenced to verify that there were no undesired mutations.

Expression and Purification of Mutant and Wild-Type Enzymes

The single mutant enzymes were obtained by co-expressing the mutated plasmid pML10 (or pMON17336) with unmutated plasmid pMON17336 (or pML10) in *E. coli* mutant strain AC70R1-504. The double mutant enzyme was obtained by co-expressing the two mutated plasmids in AC70R1-504. Mutant enzyme $S_{K198R}L_{wt}$ was expressed as previously described (6). The other mutant and wild-type enzymes were expressed in the same manner except that the concentration of IPTG was increased from 10 µM to 0.5 mM for induction. An improved procedure over the one in the previous study (6) was used for the purification of the wild-type and mutant enzymes. In the hydrophobic chromatography step, the enzyme was loaded onto the column in the presence of 1.2 M ammonium sulfate instead of 1.3 M potassium phosphate buffer. After the heat treatment step, a 50% saturation ammonium sulfate precipitation step was added, after which the pellet was dissolved in a minimal volume of extraction buffer [100 mM Hepes-NaOH, pH 8.0, 5 mM $MgCl_2$, 1 mM EDTA, and 20%(w/v) sucrose] and dialyzed against the same buffer overnight. The dialyzed enzyme was centrifuged at 20,000 g for 15 min at 4°C to remove insoluble materials before being loaded onto the DEAE Fractogel column.

SMALL SUBUNIT

	E F A E K P Q
wild-type	5′- GAA TTT GCA GAG AAA CCG CAA - 3′
	3′- CTT AAA CGT CTC TTT GGC GTT - 5′
$S_{K198R}L_{wt}$	5′- GAA TTT GCA GAG CGA CCG CAA - 3′
$S_{K198A}L_{wt}$	5′- GAA TTT GCA GAG GCA CCG CAA - 3′
$S_{K198E}L_{wt}$	5′- GAA TTT GCA GAG GAA CCG CAA - 3′

LARGE SUBUNIT

	F A E K P K G
wild-type	5′- AG TTT GCT GAA AAA CCA AAA GGT TT - 3′
	3′- TC AAA CGA CTT TTT GGT TTT CCA AA - 5′
$S_{wt}L_{K213R}$	3′- TC AAA CGA CTT GCA GGT TTT CCA AA - 5′
$S_{wt}L_{K213A}$	3′- TC AAA CGA CTT CGT GGT TTT CCA AA - 5′
$S_{wt}L_{K213E}$	3′- TC AAA CGA CTT CTT GGT TTT CCA AA - 5′

Figure 1. Nucleotide sequence and the encoded protein sequence of the potato tuber ADPGlc PPase gene in the region of Lys^{198} in the small subunit and Lys^{213} in the large subunit. The synthetic oligonucleotides used for site-directed mutagenesis at these positions are shown beside the corresponding mutants they created. The codons for position 198 in the small subunit and the anticodons for position 213 in the large subunit are underlined.

Assay of ADPGlc PPase

(A) Assay I. In the pyrophosphorolysis direction, enzyme activity was assayed according to a previously described method (9). The reaction mixture contained 80 mM glycylglycine, pH 8.0, 2 mM ADPGlc, 5 mM $MgCl_2$, 3 mM DTT, 1.5 mM ^{32}PPi (1,000-2,000 cpm/nmol), 3 mM 3PGA, 10 mM NaF, 200 μg mL^{-1} of BSA, and enzyme in a total volume of 250 μL. The assay conditions for the mutant enzymes were identical to the wild-type except that the amounts of $MgCl_2$ and ADPGlc were altered for some mutant enzymes to obtain maximal activity. The amount of $MgCl_2$ was raised to 10 mM for $S_{K198A}L_{wt}$ and $S_{K198E}L_{wt}$, and to 20 mM for $S_{K198R}L_{wt}$ and $S_{K198R}L_{K213R}$. For the $S_{K198E}L_{wt}$ and $S_{K198R}L_{wt}$ enzymes, 3 mM ADPGlc was used.

(B) Assay II. In the ADPGlc synthesis direction, enzyme activity was measured according to a previously described method (10). The reaction mixture contained 100 mM Hepes-NaOH, pH 8.0, 0.5 mM [u-^{14}C]Glc-1-P (1,000-3000 cpm/nmol), 1.5 mM ATP, 5 mM $MgCl_2$, 3.0 mM 3PGA, 3 mM DTT, 200 μg mL^{-1} of BSA, 0.3 unit of inorganic pyrophosphatase, and enzyme in a final volume of 200 μL. For assay of the $S_{K198R}L_{wt}$, $S_{K198A}L_{wt}$, $S_{K198E}L_{wt}$, and $S_{K198R}L_{K213R}$ mutant enzymes, [8-^{14}C]ATP (about 200-500 cpm/nmol) instead of [^{14}C]Glc-1-P was used to monitor the synthesis of ADPGlc, and the amount of Glc-1-P and $MgCl_2$ were increased to 30 and 20 mM, respectively, to obtain maximal activity. For the double mutant $K_{198R}L_{K213R}$ enzyme, the 3PGA concentration was increased to 10 mM in addition to the changes mentioned. Due to the high content of Glc-1-P in the assay mixture, the time for the alkaline phosphatase digestion was extended to overnight. Control experiments showed that the product [^{14}C]ADPGlc was stable during the overnight digestion.

Kinetic Studies

For determination of kinetic parameters, the concentration of the substrate or effectors tested was systematically varied with the other substrates and effectors fixed at a saturating concentration as described in *Assay I* or *II*. Kinetic data were plotted as initial velocity *versus* substrate or effector concentration. The kinetic constants, $A_{0.5}$, $S_{0.5}$, and $I_{0.5}$, which correspond to the concentration of activator, substrate, or inhibitor giving 50% of maximal activation, velocity, or inhibition, respectively, as well as the interaction coefficient, n_H, were obtained from a computer program using nonlinear iterative least-squares fitting to a modified Michaelis-Menten equation (11).

Sugar Phosphate Specificity

Reactions were performed in the ADPGlc synthesis direction (*Assay II)* with different sugar phosphates substituted for Glc-1-P. [8-^{14}C]ATP (about 200-500 cpm/nmol) was used to monitor the assay. The reaction time was extended to 30 min at 37 °C. Under the conditions with Glc-1-P as substrate, the production of ADPGlc was shown to be linear for both the wild-type and mutant enzyme $S_{K198A}L_{wt}$. The time for the alkaline phosphatase digestion was extended to 48 h. The extent of reaction was controlled by varying the amount of enzyme used in the assay.

Thermal Stability

Enzyme samples were diluted to give the same final protein concentration (0.3 mg mL^{-1}) in a final volume of 20 μL. Dilution buffer was 50 mM Hepes-NaOH, pH 8.0, 5 mM $MgCl_2$, 1 mM EDTA, 20% (w/v) sucrose, and 1 mg mL^{-1} BSA. The enzyme samples were heated for 5 min in a water bath equilibrated at 60 °C, then immediately placed on ice. The enzyme activities were assayed in the ADPGlc synthesis direction, as described in *Assay II.*

Protein Assay

Protein concentration was measured using the Pierce bicinchoninic acid (BCA) reagent (12) with BSA as the standard.

Protein Electrophoresis and Immunoblot

SDS-PAGE was performed according to Laemmli (13) on 10% polyacrylamide slab gels. Following electrophoresis, proteins on the gel were visualized by staining with Coomassie Brilliant Blue R-250 or electroblotted onto a nitrocellulose membrane (14).

After electroblotting, the nitrocellulose membrane was treated with affinity-purified rabbit anti-spinach leaf ADPGlc PPase immunoglobulin G, and the antigen-antibody complex was visualized by treatment with alkaline phosphatase-linked goat anti-rabbit IgG followed by staining with BM purple alkaline phosphatase -substrate precipitating reagent (from Boehringer Mannheim GmbH).

Results

Expression and Purification of Mutant Enzymes

Wild-type and mutant enzymes of potato tuber ADPGlc PPase were identified by immunoblotting with antibody prepared against the spinach leaf ADPGlc PPase. It had been shown that the small subunit of the potato tuber enzyme cross-reacts significantly with the anti-spinach leaf ADPGlc PPase antibody (15). Under the same conditions, the expression level of all the mutant enzymes were similar to that of the wild-type based on the results of immunoblotting. The apparent sizes of these mutant polypeptides were the same as that of the wild-type, having a molecular mass about 50 kDa. Mutant enzyme $S_{K198R}L_{wt}$ was purified to more than 85% homogeneity, as estimated from about 4 µg protein analyzed on SDS-PAGE. All the other enzymes were purified to greater than 95% homogeneity.

Kinetic Characterization of $S_{K198}L_{wt}$ Mutant Enzymes

The apparent affinity for Glc-1-P decreased dramatically when Lys^{198} in the small subunit was mutated to arginine, alanine, or glutamic acid. The $S_{0.5}$ values for Glc-1-P of the $S_{K198R}L_{wt}$, $S_{K198A}L_{wt}$, and $S_{K198E}L_{wt}$ enzymes were about 135-, 400-, and 550- fold higher than that of the wild-type enzyme, respectively (Table I). Substitution of Lys^{198} in the small subunit by alanine and glutamic acid resulted in such large $S_{0.5}$ changes that they could not be accurately determined (Figure 2A). The highest Glc-1-P amount used in the assay mixture was 40 mM, which is about 2 times higher than the $S_{0.5}$ of the alanine mutant and slightly higher than the $S_{0.5}$ of the glutamic acid mutant, due to a solubility problem. The interaction coefficients (n_H) were changed from 1.1 for the wild-type to 1.3 to 1.8 for the mutant enzymes. The changes of the V_{max} value in the synthesis direction were 2 fold or less and in pyrophosphorolysis, were less than 4 fold, except for mutant $S_{K198E}L_{wt}$. This suggests that Lys^{198} in the small subunit has virtually no role in the rate-determination step of catalysis. This is consistent with the observation on the

Figure 2. Glc-1-P dependence for the wild-type and mutant enzymes. For wild-type (●), $S_{K198R}L_{wt}$(▲), $S_{K198A}L_{wt}$(■), and $S_{K198E}L_{wt}$(○) (panel A), 100% activity corresponds to 1.2, 16.6, 33.7, 39.0 nmol/10 min, respectively. For wild-type (●) and $S_{wt}L_{K213R}$ (□) (panel B), 100% activity corresponds to 1.2 and 1.6 nmol/10 min, respectively. Initial velocities of the enzymes were determined in the ADPGlc synthesis direction (Assay II) as described under Materials and Methods with the concentration of Glc-1-P being varied. The amounts of the wild-type, $S_{K198R}L_{wt}$, $S_{K198A}L_{wt}$, $S_{K198E}L_{wt}$, and $S_{wt}L_{K213R}$ proteins were about 2.5×10^{-3}, 70×10^{-3}, 80×10^{-3}, 2.4, and 5.5×10^{-3} μg, respectively.

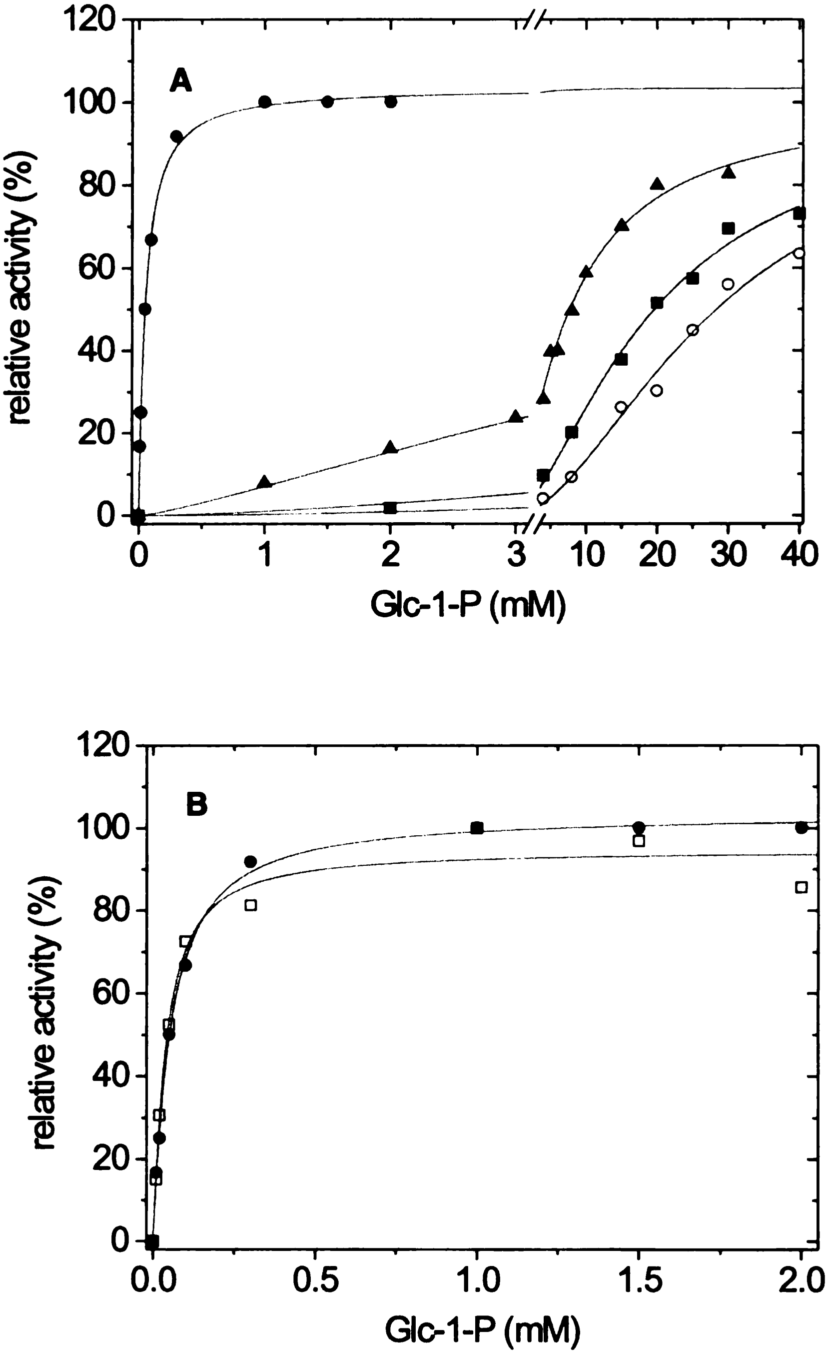
A
relative activity (%)
0
20
40
60
80
100
120
0
1
2
3
10
20
30
40
Glc-1-P (mM)
B
relative activity (%)
0
20
40
60
80
100
120
0.0
0.5
1.0
1.5
2.0
Glc-1-P (mM)

Figure 2.

corresponding Lys^{195} of the *E. coli* ADPGlc PPase (2). Replacement with glutamic acid not only caused the largest increase of $S_{0.5}$ value for Glc-1-P, but also substantially decreased the catalytic efficiency relative to the wild-type enzyme.

Considering the mutations' large effect on the $S_{0.5}$ of Glc-1-P, it was not surprising to see that the apparent affinity of ADPGlc was affected (Table I). Indeed, the $S_{0.5}$ value of ADPGlc was increased 2.5- to 10- fold. The kinetic constant for the other substrate, PPi, was increased 3- to 6- fold for the mutant enzymes (Table II). However, these changes were relatively small in comparison with the changes of the $S_{0.5}$ value for Glc-1-P. Overall, the various mutations at position 198 in the small subunit caused little or no alteration in the apparent affinities for the other substrates (ATP and Mg^{2+}) and activator (3PGA) (Table II). The data suggest that the conformations of those ligand-binding sites are relatively unchanged. The 2- to 7- fold increase of the $I_{0.5}$ value for the inhibitor, Pi, is relatively small but possibly the inhibitor site may be in close proximity to the Glc-1-P binding site.

Kinetic Characterization of $S_{wt}L_{k213}$ Mutant Enzymes

The apparent affinity for Glc-1-P was not affected when Lys^{213} in the large subunit was replaced with arginine, alanine, or glutamic acid (Table I). As shown in Figure 2B, there is no difference between the wild-type and $S_{wt}L_{K213R}$ enzyme in terms of Glc-1-P dependence. This is also true for both the $S_{wt}L_{K213A}$ and $S_{wt}L_{K213E}$ enzymes (data not shown). These observations are in sharp contrast with the effect caused by mutations on Lys^{198} in the small subunit (Figure 2A). Interestingly, although the apparent affinity of Glc-1-P was not affected, the apparent affinity for ADP-glucose decreased 2- to 4- fold (Table I). In general, mutations on Lys^{213} of the large subunit caused small changes (less than 4- fold) to the kinetic constants for the substrates (ATP, Mg^{2+}), activator (3PGA), and inhibitor (Pi) (Table II).

Table I

Comparison of the Apparent Affinity for Substrates of Potato Tuber Wild-Type and Mutant ADPGlc PPases[a]

	synthesis direction		pyrophosphorolysis direction	
	$S_{0.5}$ (n_H) for Glc-1-P (mM)	V_{max} (units/mg)[b]	$S_{0.5}$ (n_H) for ADP-glucose (mM)	V_{max} (units/mg)
wild-type	0.057 ± 0.003 (1.1)	48 ± 1	0.20 ± 0.01 (1.3)	55 ± 1
$S_{K198R}L_{wt}$	7.7 ± 0.1 (1.3)	24 ± 1	0.49 ± 0.02 (1.1)	16 ± 1
$S_{K198A}L_{wt}$	22.0 ± 2.5 (1.5)	46 ± 3	1.3 ± 0.1 (1.0)	20 ± 1
$S_{K198E}L_{wt}$	31.1 ± 2.7 (1.8)	1.7 ± 0.1	2.1 ± 0.2 (1.0)	1.4 ± 0.1
$S_{wt}L_{K213R}$	0.044 ± 0.002 (1.1)	27 ± 1	0.36 ± 0.03 (1.8)	37 ± 1
$S_{wt}L_{K213A}$	0.037 ± 0.001 (1.0)	25 ± 1	0.46 ± 0.02 (1.7)	31 ± 1
$S_{wt}L_{K213E}$	0.036 ± 0.001 (0.9)	31 ± 1	0.68 ± 0.01 (1.8)	45 ± 1
$S_{K198R}L_{K213R}$	5.6 ± 0.1 (1.5)	24 ± 1	0.72 ± 0.01 (1.9)	14 ±1

[a] Reactions were performed in either synthesis direction (*Assay II*) or pyrophosphorolysis direction (*Assay I*) as described under Materials and Methods. Data represent the average of two identical experiments ± average difference of the duplicates. The values in parentheses are the Hill interaction coefficients (n_H). [b] One unit of enzyme activity is expressed as the amount of enzyme required to form 1 μmol of ADPGlc/min at 37 °C when assayed in either synthesis or pyrophosphorolysis direction.

Table II

Kinetic Parameters of the Potato Tuber Wild-Type and Mutant ADPGlc PPases[a].

	synthesis direction				pyrophosphorolysis direction
	$S_{0.5}$ (n_H) for		$A_{0.5}$ (n_H) for	$I_{0.5}{}^{b}$ (n_H) for	$S_{0.5}$ (n_H) for
	ATP (μM)	Mg^{2+} (mM)	3PGA (mM)	Pi (mM)	PPi (μM)
wild-type	76 ± 2 (1.6)	2.2 ± 0.1 (3.7)	0.14 ± 0.01 (0.9)	1.4 ± 0.1 (1.4)	41 ± 3 (1.0)
$S_{K198R}L_{wt}$	119 ± 1 (1.5)	3.7 ± 0.1 (2.1)	0.39 ± 0.04 (1.3)	2.8 ± 0.2 (1.3)	210 ± 10 (1.5)
$S_{K198A}L_{wt}$	130 ± 4 (1.3)	2.8 ± 0.1 (2.5)	0.15 ± 0.01 (1.2)	5.0 ± 0.1 (1.1)	135 ± 5 (1.5)
$S_{K198E}L_{wt}$	102 ± 24 (1.4)	2.6 ± 0.1 (1.7)	0.07 ± 0.01 (1.3)	10.0 ± 0.2 (1.2)	240 ± 10 (1.6)
$S_{wt}L_{K213R}$	125 ± 6 (1.5)	2.0 ± 0.1 (3.6)	0.37 ± 0.01 (0.8)	0.7 ± 0.1 (1.0)	43 ± 2 (0.8)
$S_{wt}L_{K213A}$	129 ± 1 (1.4)	2.1 ± 0.1 (3.4)	0.32 ± 0.03 (1.0)	0.9 ± 0.1 (1.3)	28 ± 2 (1.5)
$S_{wt}L_{K213E}$	170 ± 8 (1.4)	1.9 ± 0.1 (3.9)	0.36 ± 0.02 (0.9)	0.7 ± 0.1 (1.2)	33 ± 1 (1.3)
$S_{K198R}L_{K213R}$	320 ± 20 (1.7)	3.5 ± 0.2 (1.8)	1.6 ± 0.1 (1.6)	1.3 ± 0.1 (1.2)	230 ± 10 (1.2)

[a] Reactions were performed in either synthesis direction (*Assay II*) or pyrophosphorolysis direction (*Assay I*) as described under Materials and Methods. Data represent the average of two identical experiments ± average difference of the duplicates. The values in parentheses are the Hill interaction coefficients. [b] The 3PGA concentration used was 3 mM for the wild type enzyme and single mutant enzymes. 10 mM 3PGA was used for the double mutant enzyme.

Kinetic Characterization of $S_{k198R}L_{k213R}$ Mutant Enzyme

When both Lys[198] in the small subunit and Lys[213] in the large subunit were replaced with arginine, the $S_{0.5}$ value for Glc-1-P was about 100-fold higher than that of the wild-type enzyme (Table I). Considering the 135-fold increase of the $S_{0.5}$ value in the mutant enzyme $S_{K198R}L_{wt}$, the double mutation did not cause a further decrease in the apparent affinity of Glc-1-P over the single mutation. In either direction of assay, the V_{max} of the double mutant enzyme is essentially the same as the single mutant enzyme, $S_{K198R}L_{wt}$.

The double mutation did not cause much alteration in the apparent affinities for Mg^{2+} and Pi (Table II). However, the $A_{0.5}$ value for 3PGA increased 11-fold relative to the wild-type. This effect seemed to be additive since the $A_{0.5}$ value for both $S_{K198R}L_{wt}$ and $S_{wt}L_{K213R}$ increased 3-fold. The 6-fold increase of $S_{0.5}$ value for PPi was similar to the effect seen in the single mutant enzyme, $S_{K198R}L_{wt}$. The other minor effects were 4-fold increases of the $S_{0.5}$ values for both ATP and ADP-glucose (Tables I and II).

Sugar Phosphate Specificity

Since Lys[198] on the small subunit was implicated in Glc-1-P binding, it was of interest to examine whether the specificity for the substrate of the $S_{K198}L_{wt}$ mutant enzymes had been changed. To analyze systematically the contribution of each specific hydroxyl group in the binding of substrate to the active site of wild-type and mutant enzymes, a variety of compounds whose sugar moieties differed from glucose stereochemically or by substitution or elimination of hydroxyl groups at different positions were used. In the measurement of the activity of mutant enzyme $S_{K198A}L_{wt}$, a large amount of enzyme had to be used to obtain accurate measurements. The results were summarized in Table III. When 6-deoxy-Glc-1-P and 6F-Glc-1-P were used to substitute for Glc-1-P, the activity of the wild-type and $S_{K198A}L_{wt}$ enzyme decreased 3.1- to 4.5- fold and 17- to 34- fold, respectively. When the other analogs were used to substitute Glc-1-P, the activity of the wild-type and $S_{K198A}L_{wt}$ enzyme decreased more than 14- and 150- fold, respectively. Thus, both enzymes showed the largest tolerance towards the elimination or substitution of the hydroxyl group at C-6 of the Glc-1-P molecule, followed by the changes at C-2, C-3 and C-4. In no case was there a substrate analog that showed a significant enhanced reactivity with the mutant enzyme as compared to the wild-type. A substitution of a carboxyl group at C-6 indicates that glucuronic acid-1-P is not a substrate. The sugar phosphate analogues also showed the same pattern of effects for the mutant enzyme $S_{K198R}L_{wt}$ (data not shown). The results indicate that the hydroxyl groups at C-2, C-3 and C-4 probably played much more important roles than the C-6 hydroxyl group in the binding process. Overall, replacement of Lys^{198} with alanine or arginine does not cause any broadened changes in substrate specificity for sugar-1-P.

Thermal Stability

After heat treatment at 60 °C for 5 min, the activity of the wild-type enzyme remained unchanged, while the $S_{K198A}L_{wt}$, $S_{K198E}L_{wt}$, $S_{wt}L_{K213A}$, $S_{K198R}L_{K213R}$ enzymes retained 104, 67, 94, and 105% activity, respectively. Thus, substitution of Lys^{198} with a negatively charged glutamic acid made the protein more susceptible to heat inactivation. Nevertheless, both residue 198 in the small subunit and residue 213 in the large subunit are not critical for the stability of the native folded state of the potato tuber enzyme.

Table III

Specificity of Sugar-phosphates as Substrates for Wild-type and Mutant $S_{K198A}L_{wt}$[a].

substrates	substrate concentration	wild-type	$S_{K198A}L_{wt}$
	mM	unit/mg	unit/mg
Glc-1-P	2	48 ± 1	1.5 ± 0.2
	10	55.8 ± 4.7	10.4 ± 0.9
6-deoxy- Glc-1-P	2	13.6 ± 0.3	0.085 ± 0.001
	10	12.5 ± 0.1	0.62 ± 0.04
6F-Glc-1-P	2	13.0 ± 0.5	0.044 ± 0.002
	10	18.1 ± 0.6	0.47[b]
2F-Glc-1-P	2	1.0[b]	0.007 ± 0.001
	10	4.0 ± 0.1	0.018 ± 0.03
mannose-1-P	2	1.7 ± 0.1	0.010 ± 0.001
	10	4.1 ± 0.2	0.058 ± 0.003
3-deoxy-glucose-1-P	2	0.5 ± 0.1	0.010 ± 0.006
	10	1.3 ± 0.1	0.017 ± 0.002
3F-Glc-1-P	2	0.05 ± 0.01	≤0.002
	10	0.15 ± 0.01	≤0.002
galactose-1-P	2	0.16 ± 0.03	≤0.003
	10	0.28 ± 0.04	≤0.002
glucuronic acid-1-P	2	≤0.01	≤0.001
	10	≤0.01	≤0.002

[a] Reactions were performed in the synthesis direction as described under Materials and Methods, with the presence of sugar-phosphates as indicated. Data represent the average of two duplications ± standard deviation. The lower limit of detection of enzyme activity for the wild-type is 0.01 unit/mg, for the mutant enzyme $S_{K198A}L_{wt}$ is 0.001 unit/mg when sufficient amount of enzyme was used in the assay as indicated in the text.

[b] Single determination.

Discussion

According to the results presented here, we can conclude that Lys^{198} in the small subunit of potato tuber ADPGlc PPase is primarily involved in Glc-1-P binding. The 135- to 550- fold increases of the $S_{0.5}$ value for Glc-1-P when this residue was replaced by other amino acids explains the high conservation of this lysine in plant and bacterial ADPGlc PPases. This lysine residue probably is required for the proper substrate binding to ADPGlc PPase under physiological concentrations of Glc-1-P. Although Lys^{198} is critical in interacting with Glc-1-P, it is obviously not essential for thermal stability. From the moderate effect on V_{max} values and the kinetic constants for ATP, Mg^{2+}, 3PGA, and Pi (Tables I and II), Lys^{198} is probably neither involved in the rate-limiting step of the catalytic mechanism nor responsible for maintaining the native conformation of the enzyme. The interaction coefficients (n_H) of Glc-1-P of the mutant enzymes were increased to 1.3-1.8 compared to 1.1 for the wild-type. However, since both the $S_{K198A}L_{wt}$ and $S_{K198E}L_{wt}$ mutant enzymes had high $S_{0.5}$ values for Glc-1-P, it was impossible to perform kinetic studies for them under saturated concentration of Glc-1-P. Only 50-70% of V_{max} could be attained based on the estimation from Lineweaver-Burk plot. Therefore, the n_H values could be overestimated for these two enzymes. Nevertheless, it has been observed for some enzymes that a single mutation that caused decreased affinity for a ligand resulted in an increase in cooperativity (16, 17). The phenomenon was explained by a theory of preexisting cooperativity (17).

As the substitution of Lys^{198} varied from basic to neutral to acidic amino acid, the apparent affinities for Glc-1-P decreased. There seems to be a highly specific requirement for a lysine residue in terms of its charge, size and shape to be present in the active site to allow optimal binding of substrate. Even the most conservative substitution of an arginine resulted in a mutant enzyme with 135-fold lower apparent affinity for Glc-1-P, suggesting that charge alone is insufficient to account for proper interaction with the substrate.

Arginine, being a slightly larger amino acid than lysine, may sterically interfere with substrate binding.

In contrast to the effects observed for the mutations of Lys^{198} on the small subunit, mutations of Lys^{213} on the large subunit had no effect on the $S_{0.5}$ of Glc-1-P. When both residues were replaced by arginine, the effect on the apparent affinity for Glc-1-P was similar to that obtained with the single arginine substitution of the small subunit, ruling out a direct role of Lys^{213} in the binding of the substrate. As indicated in the Introduction, these two lysine residues and their surrounding sequences are highly conserved in the ADPGlc PPase family. A sequence search on the large subunit of tuber ADPGlc PPase revealed no consensus sequence other than the region surrounding Lys^{213}. Therefore, it is unlikely that Glc-1-P binds to an alternative site on the large subunit. This seems to be consistent with the proposed function of this subunit, i.e., modulating the allosteric regulation of the small subunit by 3PGA and Pi, with no direct role in catalysis. It is worth noting that this lysine residue is replaced by glutamine in the large subunit of ADPGlc PPase from wheat endosperm (WE7) (18), which may reflect the relative unimportance of this residue in the large subunit. In one small-subunit isozyme, VfAGPP, of ADPGlc PPase from *Vicia faba* L. seeds, this lysine is replaced by asparagine. In the other isozyme, VfAGPC, the lysine residue is retained. Both small subunit genes are expressed in identical temporal and spatial patterns (19). However, nothing is known about the comparative kinetics of the two enzymes. Recent binding experiments on the potato tuber enzyme by equilibrium dialysis (Y. Fu, and J. Preiss, unpublished results) showed that ADPGlc bound to four sites per tetrameric enzyme. Unfortunately, experiments to determine the number of binding sites of Glc-1-P were unsuccessful due to the interference of ADP-Glc produced in the binding procedure. Still, there is a possibility that Glc-1-P may bind to the large subunit, but with no catalysis after the binding event. In any case, the data provide further evidence that the main function of the small subunit is catalysis as suggested by a previous study (6). At this stage, further studies are necessary to clarify the precise role of the large subunit.

Recently, Asp^{253} (252 in ref. 20) on the small subunit was suggested to be involved in the binding of Glc-1-P and Asp^{122} (121 in ref. 20) on the small subunit was suggested to be involved in the binding of both Glc-1-P and ATP on the basis of results obtained via random mutagenesis (20). However, replacement of both residues by asparagine resulted in relatively small changes (less than 10-fold) of the $S_{0.5}$ of the substrates compared to the effect seen with mutations of Lys^{198}. It is possible that those mutations may affect directly or indirectly the conformation of the substrate domain rather than disrupting specific interactions between the enzyme and the substrates.

The Lys^{195} region (FVEKP) of *E. coli* ADPGlc PPase is not only conserved in potato tuber ADPGlc PPase (FAEKP), but also identical to the mannose-1-P binding site of PMI/GMP (21). Furthermore, this motif or a closely related sequence (GVE$\underline{K}$P, IVE$\underline{K}$Y, KVI$\underline{K}$P, FKE$\underline{K}$P) is found in many enzymes with the common characteristic of catalyzing the synthesis of a nucleotide diphosphate-sugar from sugar phosphate and nucleotide triphosphate (Table IV). Therefore, FVEKP may be part of a sugar phosphate-binding motif for this class of sugar nucleotide pyrophosphorylases. Of course, other sequences must dictate the sugar specificity, e.g. for Man-1-P or Glc-1-P.

Various sugar-1-P analogs whose sugar moieties differed from Glc at each hydroxyl group were tested as substrates for wild-type and mutant enzyme $S_{K198A}L_{wt}$ (Table III). No broadened specificity for the mutant enzyme was observed. This probably suggests that Lys^{198} only participates in forming an ionic bond between its positively charged ε-amino group and the negatively charged phosphate group of Glc-1-P. Those hydroxyl groups may interact with the side chains of the other residues in the active site, i.e. by hydrogen bonding, to anchor the substrate correctly. Therefore, those analogs tested would have similar effects on the wild-type as well as the mutant enzymes.

Table IV

Conservation of the Sugar Phosphate Binding Motif in Sugar Nucleotide Pyrophosphorylases

Enzymes	source	sugar-P binding motif[a]	references
ADPGlc PPase	*E. coli*	IIEFVEKPAN	2
PMI/GMP	*P. aeruginosa*	VQS*****DE	21
PMI/GMP	*X. campestri*	VER*****LA	22
GDP-mannose PPase	*E. coli*	VRT*****NL	23
GDP-mannose PPase	*S. typhimurium*	VAE*****DI	24
UDP-glucose PPase	*E. coli*	PMVG****KA	25
UDP-glucose PPase	*B. subtilis*	VKN*****PK	26
UDP-glucose PPase	*Solanum tuberosum* L.	TLKI***Y**	27
UDP-glucose PPase	*D. discoideum*	ETNK*I**YK	28
CDP-glucose PPase	*Y. pseudotuberculosis*	VRS*K***KG	29

[a] Lys-195 in *E. coli* ADPGlc PPase and Lys-175 in *P. aeruginosa* PMI/GMP, which were shown to bind to Glc-1-P and mannose-1-P respectively, are underlined. * Signifies the same amino acid as in the *E. coli* ADPGlc PPase sequence.

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CHAPTER 4

MECHANISM OF REDUCTIVE ACTIVATION OF POTATO TUBER ADP-GLUCOSE PYROPHOSPHORYLASE

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Abstract

The catalytic activity of potato tuber (*Solanum tuberosm* L.) ADP-glucose pyrophosphorylase is activated by a preincubation 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 by 5, 5'-dithiobis(2-nitrobenzoic acid) quantitation. By analyzing the activated and nonactivated enzymes on SDS-PAGE under nonreducing condition, 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). Preincubation 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 instaneous activation rates as found in the wild-type in the reduced state. A two-step activation model is proposed.

Introduction

Plant ADPGlc PPases are mainly regulated by 3PGA and Pi. However, little information is known on the possible activation of ADPGlc PPases via reduction. It is well established that the activity of several chloroplastic enzymes is regulated by reversible thiol/disulfide interchange (1, 2). During photosynthetic electron transport in the light, covalent redox modification mediated by a redox chain, the ferredoxin-thioredoxin system, leads to reductive activation of several stromal target enzymes, e.g., fructose-1,6-bisphosphatase, NADP-malate dehydrogenase, phosphoribulokinase, etc. (3). The activity of potato tuber ADPGlc PPase was found to be stimulated by dithiothreitol (DTT) (4, 5). The authors had suggested 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, I 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

Reagent

ATP, ADPGlc, Glc-1-P, 3PGA, inorganic pyrophosphate, and 4-vinylpyridine were purchased from Sigma. ^{32}PPi were purchased from DuPont New England Nuclear. [^{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 before (6). By a described procedure (6), the wild-type enzyme was purified to apparent homogeneity as estimated from about 4 μg protein on SDS-PAGE. The mutant proteins were purified as the wild-type, except that the heat treatment and the hydrophobic chromatography steps were eliminated, and a second Mono Q chromatography step was added. For the added Mono Q step, protein elution was carried in 50 mM Hepes, pH 8.0, 5 mM $MgCl_2$, 1 mM EDTA, and 20% sucrose using a linear NaCl gradient from 0.15 M to 0.30 M over 20 min. The mutant enzymes were purified to about 50% homogeneity as estimated by SDS-PAGE with about 3 μg of protein.

Assay of ADPGlc PPase

(A) Assay I. In the pyrophosphorolysis (reverse) direction, enzyme activity was assayed according to the method of Morell et al. (7). The reaction mixture contained 80 mM glycylglycine, pH 8.0, 2 mM ADP-glucose, 5 mM $MgCl_2$, 3 mM DTT, 1.5 mM ^{32}PPi (about 1,000-2,000 cpm/nmol), 10 mM NaF, 200 μg/mL of bovine serum albumin, and enzyme in a total volume of 250 μl. This assay was primarily used for measuring enzyme activity during purification steps.

(B) Assay II. In the ADP-glucose synthesis (forward) direction, enzyme activity was measured at 37 °C according to the method of Preiss *et al.* (8). Enzyme activity was measured in this direction in the kinetic analysis unless otherwise indicated. The reaction

mixture contained 100 mM Hepes-NaOH, pH 8.0, 0.5 or 1 mM [^{14}C]Glc-1-P (1,000-3000 cpm/nmol), 1.5 mM ATP, 5 mM $MgCl_2$, 3 mM DTT, 200 μg/mL of bovine serum albumin, 0.3 unit of inorganic pyrophosphatase, and enzyme in a final volume of 200 μL. The reaction time was 1 min unless otherwise indicated.

Reductive Activation of ADPGlc PPase

The enzyme was activated with mixture A (100 mM Hepes, pH 8.0, 2 mM ADPGlc, and 3 mM DTT) at 37 °C for 30 min unless otherwise indicated. This condition was referred as activation condition. In the control, the enzyme was incubated with mixture B (100 mM Hepes, pH 8.0, and 2 mM ADPGlc). This condition was referred as 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 μg) were incubated either under 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 A_{412nm} was measured every 15 s with a Beckman spectrophotometer model DU680. A 2-nitro-5-thiobenzoate extinction coefficient of 13, 600 $M^{-1}cm^{-1}$ was used for the calculation (9). A molecular mass of 202 kDa (10) was used to calculate the amount of enzyme used for DTNB measurement. The desalting procedure could efficiently remove DTT from the samples as judged by the fact that in the absence of enzyme both mixture A and mixture B gave the same blank reading after desalting. After removal of DTT, the newly formed –SH groups of the activated enzyme were retained as determined by SDS-PAGE under nonreducing condition. 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. (11).

Determination of the Reduction and Activation Time Course

Enzyme (48 µg) was incubated with mixture A at 37 °C in a final volume of 48 µL. Aliquots of 2.5 µL were withdrawn periodically for activity measurement in the synthesis direction, in parallel, aliquots of 4 µL were withdrawn and immediately mixed with 4 µL 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 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 Laemmi (12) on 10% polyacrylamide gel. 2-mercaptoethanol was not added to the protein samples under nonreducing condition. The nonactivated potato tuber ADPGlc PPase (8 µg) was separated in SDS-PAGE under nonreducing condition 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 (13).

For immunoblot analysis, proteins were transferred to a nitrocellulose membrane 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 alkaline phosphatase -substrate precipitating reagent (Boehringer Mannheim GmbH).

Determination of the Total Number of Disulfides

Potato tuber ADPGlc PPase (17 µg) was incubated with mixture A (activation condition) or mixture B (nonactivation condition). 8M urea was added and the samples were incubated 15 min at 50 °C then 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 (TCA), 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 then 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

(A) Direct Labeling with [^{14}C]Iodoacetic Acid. Potato tuber ADPGlc PPase (216 μg) 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% TCA.

(B) Reverse Labeling. Potato tuber ADPGlc PPase (68 μg) 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 then 1 h at 37 °C. Samples were brought to room temperature and incubated 2 h. After precipitation with 10% TCA, the pellets were washed four times and dissolved with 25 μl 8 M urea in 0.4 M NH_4HCO_3; 5 μl of 24 mM DTT was added and samples were incubated at 50 °C for 15 min then 37 °C for 30 min. After cooling down to room temperature, 10 μl 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 μl of 100 mM DTT.

(C) 4-Vinylpyridine Labeling. Potato tuber ADPGlc PPase (17 μg) was incubated either under activation or nonactivation conditions in a final volume of 15 μl. Then 2.8 μl 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.

For potato tuber ADPGlc PPase after direct labeling with [^{14}C]iodoacetic acid, trypsin digestion was performed according to a described procedure (14). For digestion of potato tuber enzyme after reverse labeling, water was added to adjust the final concentration of urea to 2 M and then TPCK-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. 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 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 to 45% solvent B) and a 90-min linear gradient (5 to 30% solvent B) was used, respectively, at a flow rate of 40 μl/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 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) (15); the $S_{C12A}L_{wt}$ mutant was obtained by sequential polymerase chain reaction steps (16). 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'-CGCAGAATTCACAGACA$\underline{\text{TCT}}$CTAGACCCAGATGC- 3'
3'-GCGTCTTAAGTGTCTGT$\underline{\text{AGA}}$GATCTGGGTCTACG- 5'
$S_{C12A}L_{wt}$ 5'-ATTCACAGACA$\underline{\text{GCT}}$CTAGACCCAGATGCTAGCCGGAGT- 3'
3'-TAAGTGTCTGT$\underline{\text{CGA}}$GATCTGGGTCTACGATCGGCCTCA- 5'

Results

I. Reductive Activation of Potato Tuber ADPGlc PPase

The catalytic activity of potato tuber ADPGlc PPase was found to increase with time (showing nonlinear kinetics) when measured in the absence of activator, 3PGA. Various combinations of effectors were tested for their ability to activate the enzyme during a preincubation at 37 °C.

Activation of Potato Tuber ADPGlc PPase by ADPGlc and DTT

As seen in Figure 1, 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 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 the catalysis. Experiment 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 affinity of Ca^{2+} for the enzyme ($S_{0.5}$= 1.8 mM; Y. Fu, and J. Preiss, unpublished results) 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

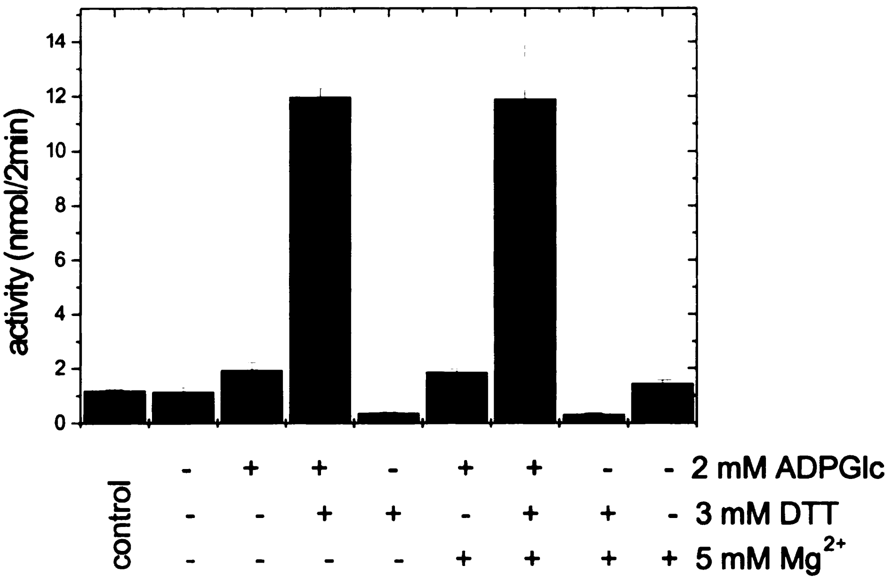

Figure 1. Activation of potato tuber ADPGlc PPase by ADPGlc and DTT. Enzyme (1.7 μg) was incubated with 100 mM Hepes, pH 8.0, 0.2 mg/mL BSA, and different additional effectors in a final volume of 8 μl for 30 min at 37 °C. The synthesis reaction was started by adding 192 μl assay mixture into the incubated solution and continued at 37 °C for 2 min. Control experiment was carried out without adding additional effectors and the preincubation step was omitted.

efficiency. After the incubation, 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 (2, 17). As shown in Figure 2, the enzyme was only activated when all three effectors were present at the same time. With DTT present in cases where the three effectors were not present together, the enzyme activity actually decreased from the control value after a 30-min preincubation.

Since 9.6 nmol of ADPGlc was produced when all three effectors were present in the preincubation (see Figure 2 legend), another experiment was conducted to differentiate the activation from the formed ADPGlc and that from ATP, Glc-1-P and Ca^{2+}. Figure 3 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, Ca^{2+} could activate potato tuber ADPGlc PPase without prior formation of ADPGlc.

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 condition. As shown in Figure 4, the activation of potato tuber ADPGlc PPase was accompanied by the appearance of about 2.5 new sulfhydryl groups per tetrameric enzyme. 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 condition (Figure 5, lanes a and b). The activated protein migrated as a single band with molecular mass about50 kDa (Figure 5, lane a). This was in agreement with previous studies on native

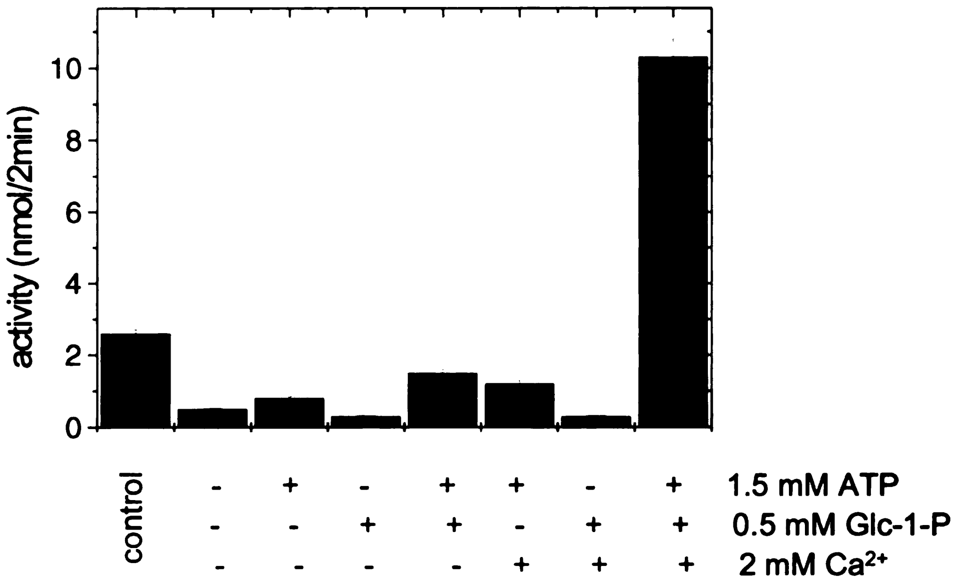

Figure 2. Activation of potato tuber ADPGlc PPase by ATP, Glc-1-P, Ca^{2+} and DTT. Enzyme (2 μg) was preincubated with a mixture that contained 80 mM glycylglycine, pH 8.0, 0.2 mg/mL BSA, 3 mM DTT and different combinations of additional effectors in a final volume of 80 μl for 30 min. The synthesis reaction was started by adding 60 μl incubated solution to 140 μl of assay mixture and continued at 37 °C for 2 min. A control experiment was carried out without adding additional effectors and the activation step was omitted. Since the enzymatic reaction would slowly take place when three effectors were present in the preincubation, the value (10.3 nmol/2min) 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.

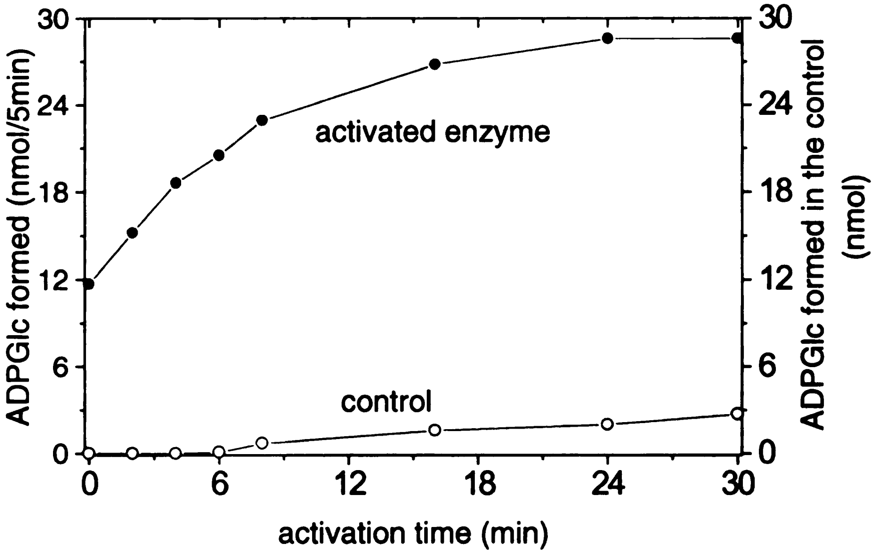

Figure 3. Activation of potato tuber ADPGlc PPase by ATP, Glc-1-P, Ca^{2+} and DTT with different incubation times. Enzyme (22.1 μg) was activated with a mixture containing 80 mM glycylglycine, pH 8.0, 0.2 mg/mL BSA, 3 mM DTT, 1.5 mM ATP, 2 mM $CaCl_2$ and 0.5 mM $[^{14}C]$Glc-1-P (942 cpm/nmol) at 37 °C in a final volume of 1,360 μl. An 80 μl aliquot was withdrawn periodically to measure the activity in the synthesis reaction for 5 min, in parallel, an 80 μl 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.

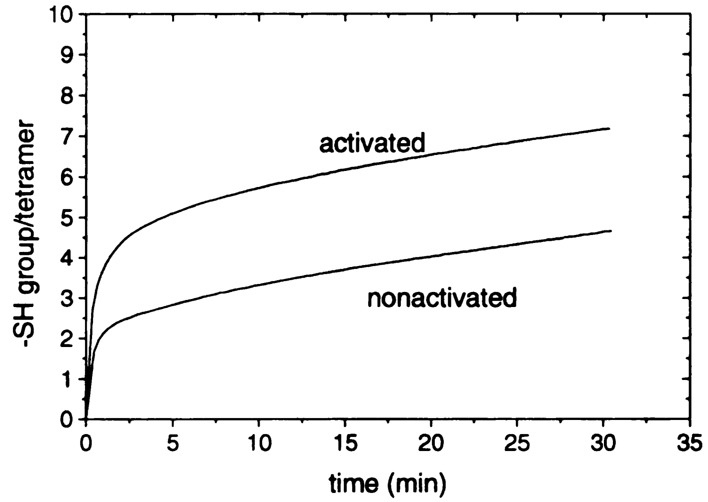

Figure 4. DTNB determination of the available sulfhydryl groups of the activated and nonactivated potato tuber ADPGlc PPase. The experiment was performed as described under MATERIALS AND METHODS.

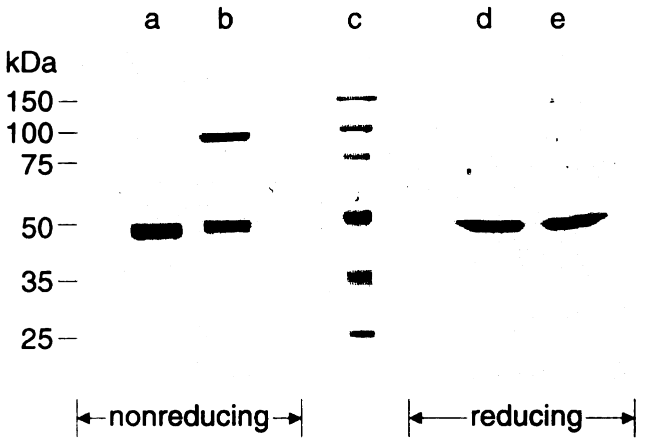

Figure 5. Electrophoretic analysis of the activated and nonactivated potato tuber ADPGlc PPase. Enzyme (36 μg) was incubated under either activation or nonactivation conditions as described under MATERIALS AND METHODS. About 1.5 μg of activated (lanes a and d) or nonactivated enzymes (lanes b and e) 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.

and cloned ADPGlc PPase (10, 18) that showed the molecular masses of the small and large subunit were 50 and 51 kDa, respectively. Apparently, the small and large subunits were too close to be distinguishable. The nonactivated enzyme migrated as two bands corresponding to molecular masses of 50 and 100 kDa (Figure 5, 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 (10), and the latter was the same as that of the large subunit deduced from cDNA sequence (18) 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 (Figure 5, lane 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) (Figure 5), 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 result was shown in Figure 6. The rate of enzyme activation matched closely the rate of reduction. It shows a correlation between the reduction and the activation process, and indicates 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.

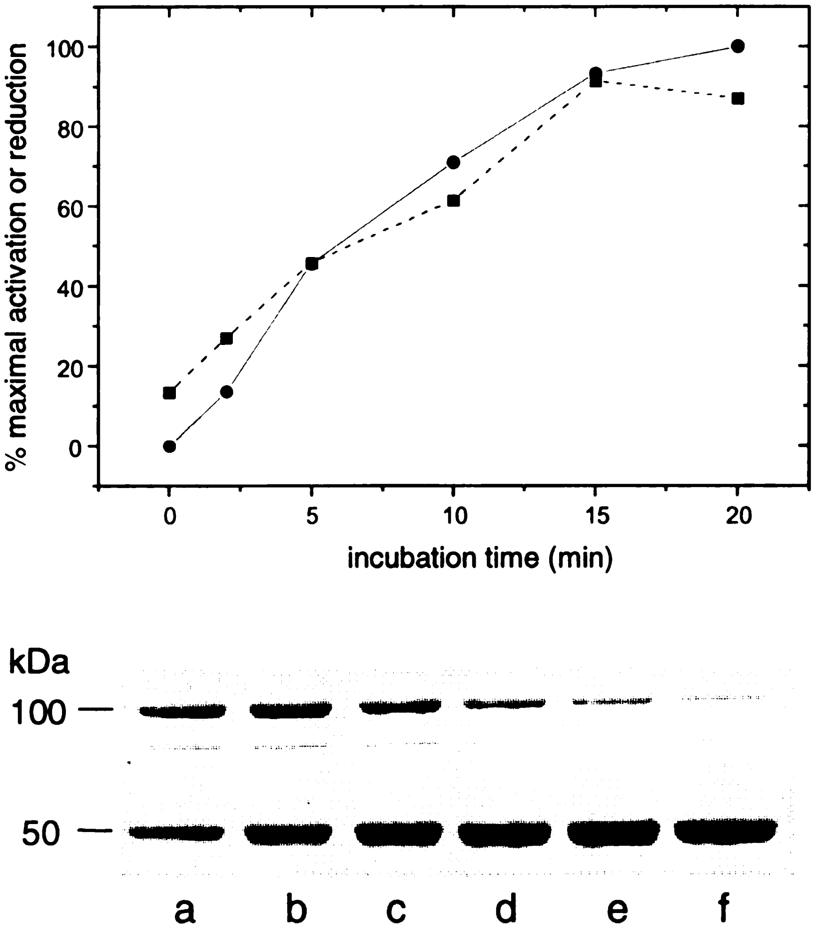

Figure 6. Time course of potato tuber ADPGlc PPase activation (•) and reduction (■). Activation was performed in mixture A (100 mM Hepes, pH 8.0, 2 mM ADPGlc, and 3 mM DTT). Aliquots were withdrawn periodically for activity measurements and, in parallel, for SDS-PAGE analysis. The enzyme activities corresponding to 0 and 100% activation were 1.7 and 9.8 nmol/min, respectively.

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 Figure 7A, the potato tuber 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 instaneous activation. The enzyme reached 80% of the maximal activity within 10 sec. This is in agreement with the result in Figure 6, which shows 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 Figs. 1 and 2. When the enzyme was first incubated with ADPGlc, subsequent addition of DTT could not activate the enzyme in the absence of ADPGlc (Figure 7B). 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.

Reversibility of the Reductive Activation

When the activated (reduced) form (Figure 8A, lane b) of enzyme was incubated with DTNB, a mobility shift of the 50-kDa band to 100-kDa position could be observed on SDS-PAGE under nonreducing condition (Figure 8A, lane c). Oxidation by DTNB was accompanied by a decrease of the enzyme activity (Figure 8B), suggesting the reformation of the intermolecular disulfide bridge. The DTNB treated enzyme

Figure 7. 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 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 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 (O). 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.

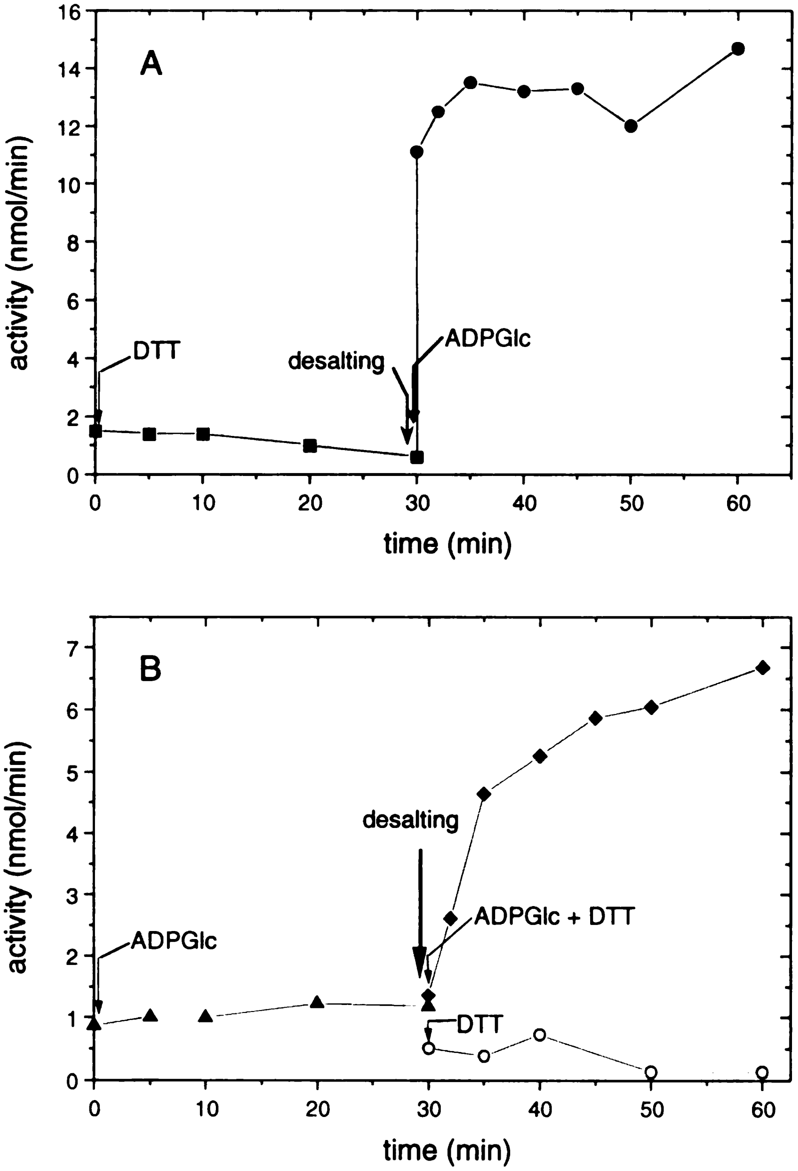
A
activity (nmol/min)
0
2
4
6
8
10
12
14
16
DTT
desalting
ADPGlc
time (min)
0
10
20
30
40
50
60
B
activity (nmol/min)
0
1
2
3
4
5
6
7
ADPGlc
desalting
ADPGlc + DTT
DTT
time (min)
0
10
20
30
40
50
60

Figure 7.

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

Figure 8. Reversibility of the reductive activation of potato tuber ADPGlc PPase. In the control, 38 μg of enzyme was mixed with 100 mM Hepes, pH 8.0 and 2 mM ADPGlc in a final volume of 80 μl. Two μl 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 μl at 37 °C for 30 min. Aliquots of about 1.2 μg enzyme were withdrawn at different stage to measure the activity in the synthesis direction (B), in parallel, aliquots of about 4.8 μg enzyme were withdrawn for SDS-PAGE analysis under nonreducing condition (A).

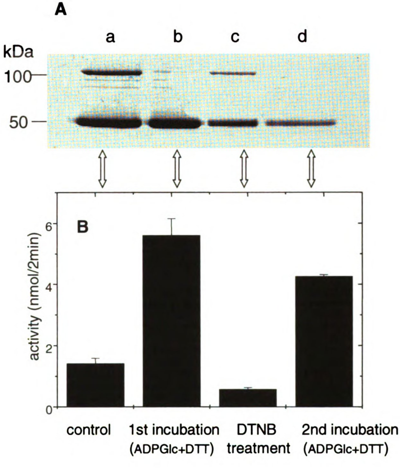
A
a
b
c
d
kDa
100
50
B
activity (nmol/2min)
0
2
4
6
control
1st incubation
(ADPGlc+DTT)
DTNB
treatment
2nd incubation
(ADPGlc+DTT)

Figure 8.

could be reactivated by a second preincubation with ADPGlc and DTT. Upon reactivation, the intermolecular disulfide bridge was reduced again (Figure 8A, lane d).

II. Identification of the Intermolecular Disulfide Bridge

Determination of the Total Number of Disulfide Bridge

There are a total 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.

Direct Labeling with [^{14}C]iodoacetic acid

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 Figure 9, 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 labeling (Figure 9).

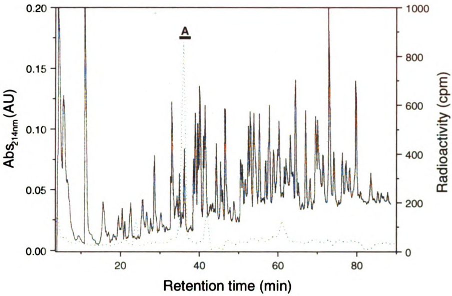

Figure 9. HPLC separation of tryptic fragments from the activated and ^{14}C-carboxymethylated potato tuber ADPGlc PPase. Elution was monitored by the UV absorption at 214 nm (solid line), and the radioactivity was determined by withdrawing 200 μl aliquots from each fraction and measured by liquid scintillation 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.

Table II

Sequence Analysis of the $[^{14}C]$Iodoacetic Acid Labeled Tryptic Peptides and 4-Vinylpyridine Labeled ADPGlc PPases[a]

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

[a] A and B refer to purified labeled fractions of the HPLC chromatograms of Figs. 9 and 10; 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 (10, 19). Boldface sequences were assigned to the small subunit. The position number of the first amino acid of peptide A refers to the sequence of the small subunit (10). The labeled cysteine residues were underlined: Cm-C, carboxymethylcysteine; PE-C, *s*-β-(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. [b] Besides the major sequence shown in the table, there was also a minor sequence corresponding to Ala^{196}-Lys^{208} in the small subunit. [c] X indicates that no amino acid could be detected in the cycle.

Reverse Labeling with [^{14}C]iodoacetic acid

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, which would specifically label the oxidized (disulfide) groups, was performed for the nonactivated enzyme. As shown in Figure 10, 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 carboxymethylcysteine was identified at cycle 11. This is in agreement with the result from Figure 9. 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 was observed (data not shown).

4-vinylpyridine Labeling

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 results were expected to show 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. On the contrary, 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 cystine 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

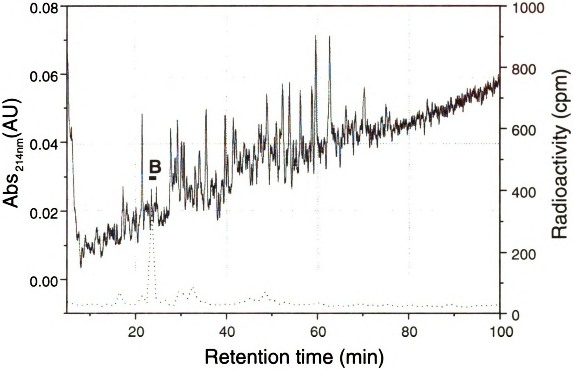

Figure 10. HPLC separation of tryptic fragments of the nonactivated potato tuber ADPGlc PPase after reverse labeling with [^{14}C]iodoacetic acid. The details are the same as described in the legend of Figure 9.

disulfide bridge in the nonactivated (oxidized) potato tuber ADPGlc PPase. The disulfide was cleaved 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 (18). The two mutant enzymes, $S_{C12S}L_{wt}$ and $S_{C12A}L_{wt}$, were produced at a similar level to the wild-type based on the result of 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 a nitrocellulose membrane. 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 (Figure 11). Under reducing condition, the mutant and wild-type enzymes migrated as single bands. 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 Figure 12, substitution of Cys^{12} in the small subunit by either Ser or Ala eliminated the requirement of DTT on the activation of potato tuber ADPGlc PPase. Another striking difference between the mutant enzymes and wild-type was the time course of activation. The wild-type needed 17.5 min to reach maximal activity, the two mutant enzymes were fully activated within 10 seconds. Thus, the mutant enzymes show the same activation characteristics as the reduced wild-type (Figure 7A)

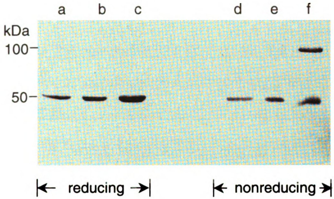

Figure 11. Immunoblotting analysis of wild-type and mutant potato tuber ADPGlc PPases. Wild-type (*lanes c and f*), $S_{C12S}L_{wt}$ (*lanes a and d*), and $S_{C12A}L_{wt}$ (*lanes b and e*) were subjected to SDS-PAGE under reducing (*lanes a-c*) and nonreducing (*lane d-f*) conditions and transferred to a nitrocellulose membrane. Immunoreactive species were detected using antibody against spinach leaf ADPGlc PPase.

Figure 12. 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 reaction on aliquots of 20 µl as a function of time. 100% activity of the wild-type, $S_{C12S}L_{wt}$, and $S_{C12A}L_{wt}$ enzymes corresponds to 16.6, 8.7, and 10.3 nmol/min, respectively. Arrows indicated the addition of ADPGlc or ADPGlc plus DTT.

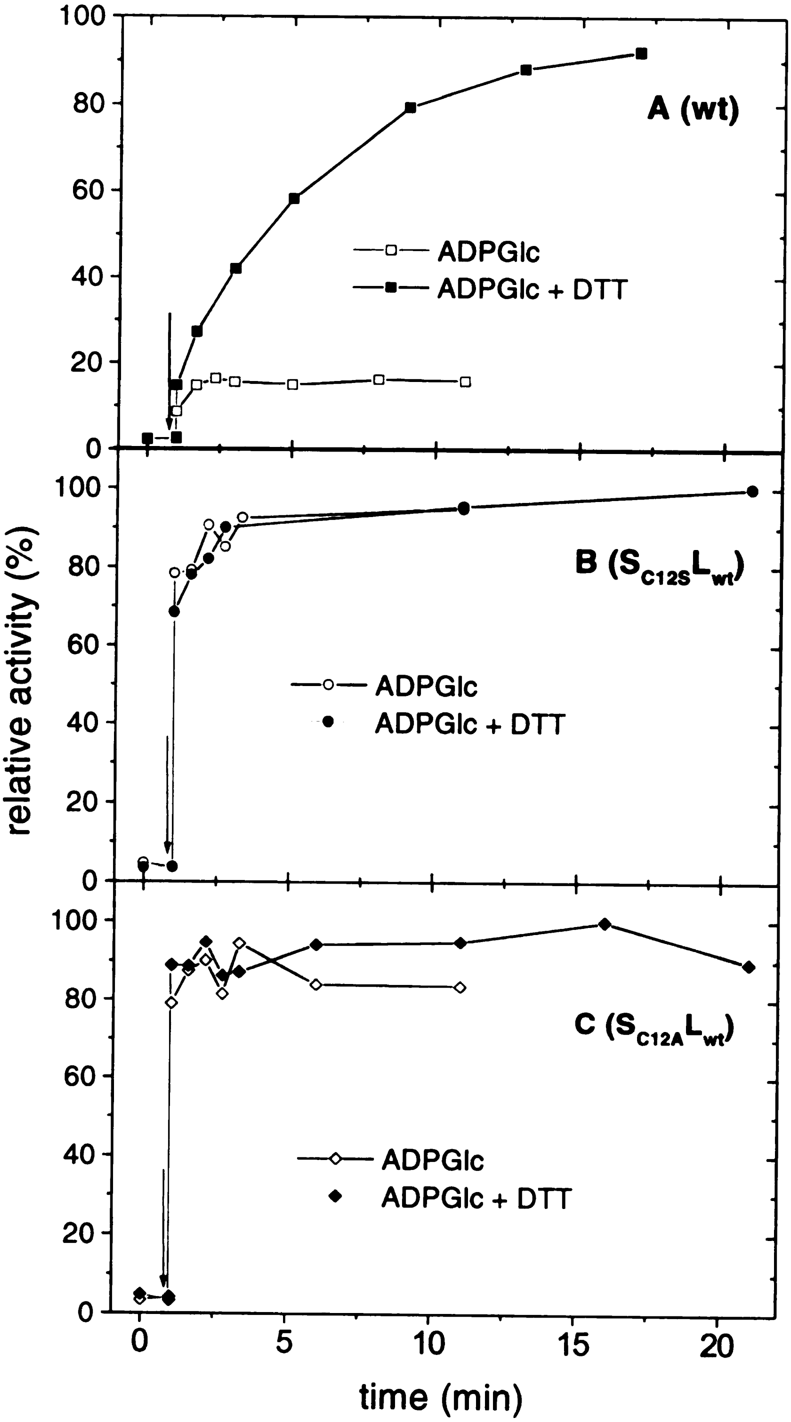
A (wt)
—□— ADPGlc
—■— ADPGlc + DTT
B ($S_{C12S}L_{wt}$)
—○— ADPGlc
● ADPGlc + DTT
C ($S_{C12A}L_{wt}$)
—◇— ADPGlc
◆ ADPGlc + DTT
relative activity (%)
time (min)
0
20
40
60
80
100
0
5
10
15
20

Figure 12.

Discussion

The present work shows that the activation of the potato tuber ADPGlc PPase proceeds via a reduction of the intermolecular disulfide bridge between its small subunits and subsequent conformational change induced by 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* (20), *R. rubrum* (21), and barley leaf (22). 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 (20). Therefore, all ligands, 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. By analyzing protein samples withdrawn from different time points during 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 Figure 6 and Figure 7 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 (23). However, inclusion of ADPGlc did not accelerate the reduction rate of potato tuber ADPGlc PPase (data not shown). This further indicates that the activation of potato tuber enzyme proceeds in 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 (9). DTNB treatment reversed the activation of potato tuber ADPGlc PPase by reoxidizing the reduced intermolecular disulfide bridge (Figure 8). Addition of ADPGlc and DTT reactivated the enzyme. These results suggested 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 a disulfide in ADPGlc PPase, the stoichiometry that 1 mole of 2-nitro-5-thiobenzoate anion was formed per mole 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 correctly folded state. The potato tuber ADPGlc PPase became unstable when DTT was present in the preincubation mixture (Figs. 1, 2 and 3). 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, the reduced wild-type and mutant enzymes were thermal labile at 60 °C for 5 min, while the wild-type enzyme was stable at this condition (10).

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 (B. Smith-White, and J. Preiss, unpublished results). 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 (Y. Fu, and J. Preiss, unpublished results). Reduction of this disulfide bridge made the spinach leaf enzyme heat labile as 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 chloroplastic enzymes are regulated by reversible thiol-disulfide interchange mediated by light controlled ferredoxin-thioredoxin system (2). Interestingly, the same potato ADPGlc PPase small subunit gene is expressed both in tubers (non-photosynthetic tissue) and leaves (photosynthetic tissue) (24). 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. Reduced thioredoxin from *Spirulina* was substituted for DTT to activate the potato tuber ADPGlc PPase, however, no significant effect could be observed. There is still a possibility that a wrong type of thioredoxin was used. 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.

In summary, the activation mechanism of potato tuber ADPGlc PPase is shown in Figure 13. The intermolecular disulfide bridge between the small subunits locks the protein in the 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 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.

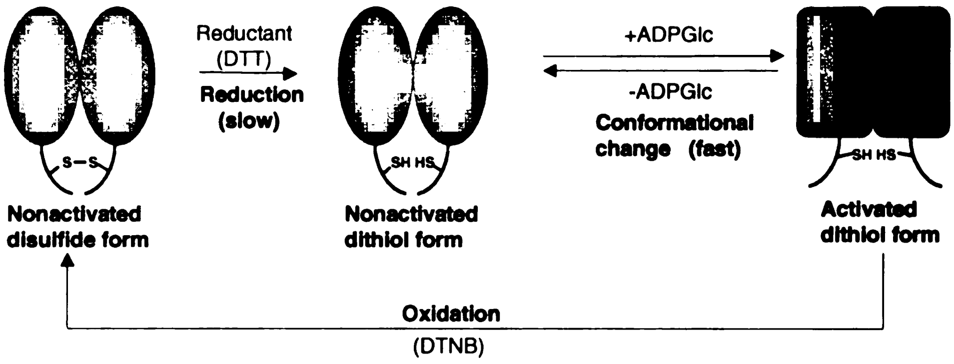

Figure 13. A proposed model for the reductive activation mechanism of potato tuber ADPGlc PPase. Only the small subunits are shown. The amino-terminal extensions are shown on the outside of each subunit.

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CHAPTER 5

SUMMARY AND PERSPECTIVES

SUMMARY AND PERSPECTIVES

1. Summary

The expression of the full-length cDNA encoding the small subunit of potato tuber ADPGlc PPase in *E. coli* results in a catalytically active enzyme. After purifying to homogeneity, its specific activity (51.6 units/mg) is close to that of the purified native (56.9 units/mg; ref. 1) and cloned (63 units/mg) heterotetrameric potato tuber enzyme. In contrast, the activity of the large subunit is negligible (<0.001 units/mg). The major differences between the homotetrameric small subunit and the heterotetrameric enzyme are their allosteric regulatory properties by 3PGA and Pi. The small subunit requires much higher concentrations of 3PGA for maximal activation and is more sensitive to inhibition by Pi. The kinetic constants for the substrates are similar between the small subunit and the heterotetrameric enzyme. These observations suggest that the small subunit is mainly involved in catalysis and the large subunit functions to modulate the sensitivity of the small subunit toward allosteric regulation.

The N-terminus of the small subunit is likely involved in the regulatory properties and heat stability of the heterotetrameric enzyme since these properties are altered in a recombinant enzyme that contains the truncated small subunit (at the N-terminus) and the large subunit (2). As a mater of fact, it is the N-terminal intermolecular disulfide bridge between the small subunits that is responsible for the heat stability of the enzyme (Chapter 4). The expression system with the full-length small subunit and large subunit provides a useful tool to identify important residues or domains in the catalysis and regulation of the holoenzyme.

The lysine residues corresponding to Lys^{198} in the small subunit and Lys^{213} in the large subunit of the heterotetrameric potato tuber enzyme are highly conserved in the homotetrameric bacterial enzymes, and in both the small and large subunits of plant ADPGlc pyrophosphorylases. The conserved lysine residue (Lys^{195}) in the homotetrameric *E. coli* enzyme was shown to be involved in the binding of the substrate, Glc-1-P (3). The functions of the conserved lysine residues in the two subunits of the heterotetrameric potato tuber enzyme were investigated by site-directed mutagenesis methods. The apparent affinity for Glc-1-P of the wild-type enzyme decreased 135-, 400-, and 550- fold by replacing Lys^{198} of the small subunit with arginine, alanine, and glutamic acid, respectively. The results suggest that both the charge and size of this residue influence Glc-1-P binding. Those mutations had little effect on the kinetic constants for the other substrates (ATP and Mg^{2+} or ADP-glucose and PPi), activator (3PGA), inhibitor (Pi), and on the thermal stability. Experiments to substitute Glc-1-P with various sugar-1-P analogs indicates that the hydroxyl groups at C-2, C-3 and C-4 of the glucose unit played much more important roles than the C-6 hydroxyl group in the binding process. Replacement of Lys^{198} with either alanine or arginine does not cause any broadened changes in substrate specificity for sugar-1-P. These observations suggest that Lys^{198} probably only participates in forming an ionic bond between its positively charged ε-amino group and the negatively charged phosphate group of Glc-1-P.

In contrast to the effects seen from mutations on the conserved residue (Lys^{198}) of the small subunit, mutagenesis of the conserved residue (Lys^{213}) in the large subunit had no effect on the apparent affinity for Glc-1-P by substitution with arginine, alanine or glutamic acid. A double mutant, $S_{K198R}L_{K213R}$, was also obtained with a 100-fold reduction of the apparent affinity for Glc-1-P. This effect is similar to that from the single arginine mutation on the small subunit. These findings indicate that Lys^{198} in the small subunit is directly involved in the binding of Glc-1-P, while Lys^{213} in the large subunit is not. This is consistent with the proposed function of the two subunits as mentioned in the preceding text.

Chapter 4 focuses on the study of the mechanism of reductive activation of heterotetrameric potato tuber enzyme. The intermolecular disulfide bridge between the small subunits locks the protein in the 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 to its nonactivated dithiol form, while reoxidation of the intermolecular disulfide bridge converted the enzyme back to its nonactivated disulfide conformation. Therefore, the activated dithiol form and the nonactivated disulfide form of the enzyme could be interconverted.

Both chemical modification and site-directed mutagenesis approaches were used to locate the intermolecular disulfide bridge between the small subunits. Analysis of the tryptic digest of the activated and ^{14}C-carboxymethylated potato tuber enzyme revealed that the radioactive label was mostly incorporated in Cys^{12} of the small subunit. The same sulfhydryl was found to be labeled for the nonactivated enzyme by a reverse labeling procedure. These results were further confirmed by N-terminal sequence analysis of 4-vinylpyridine labeled potato tuber ADPGlc PPase. *S*-β-(4-pyridylethyl)-cysteine was identified at position 12 in the small subunit of the activated enzyme, while it was not detected in the same position in the small subunit of the nonactivated 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. These findings indicate that the intermolecular disulfide bridge is located between Cys^{12} of the small subunits of the potato tuber ADPGlc PPase.

2. Perspectives

Although the small subunit is mainly implicated in catalysis and the large subunit functions to modulate the regulatory properties of the small subunit, the precise roles of the two subunits are not well understood. The C-terminal region of the large subunit of the enzymes from both maize endosperm and potato tuber was found to be important for allosteric regulation (4). However, recent mutagenesis studies showed that the putative activator sites in the small subunit play more important roles in the interaction of the activator with the potato tuber enzyme than the homologous sites in the large subunit (5). Therefore, the small and large subunits do not simply act as catalytic and regulatory subunits, respectively, in the holoenzyme. Although mutagenesis experiments have provided very useful information concerning the structure-function relationships of ADPGlc PPase, the best approach is to crystallize the enzyme and determine its three-dimensional structure. By comparison of the three-dimensional structure of the homotetrameric small subunit with that of the heterotetrameric holoenzyme, the function and the interaction of the two subunits can be understood to a much greater extent. It can also give insight into the regulatory mechanism of the enzyme. This can be quite beneficial since the structure of the potato tuber enzyme can be viewed as a model of those of the other plant ADPGlc PPases. It is reasonable to expect that the current basic research will be transformed into future improvement of starch content in plants, especially in the storage organs of the major crops

Many enzymes known to be activated by DTT were later found to be modulated by thioredoxin, for example, NADP-glyceraldehyde-3-phosphate dehydrogenase, fructose-1,6-bisphosphatase, sedoheptulose-1,7-bisphosphatase, phosphoribulokinase, and NADP-malate dehydrogenase (for review, see ref. 6). Although reduced thioredoxin from *Spirulina* could not activate the potato tuber enzyme, this may be simply due to a specificity problem. Different types of thioredoxin, e.g., thioredoxin-f or thioredoxin-m, from different sources could still be tested on the activation of the potato tuber enzyme.

In vivo, ADPGlc PPase is mainly under the allosteric regulation by 3PGA and Pi. It would be of interest to characterize the kinetic constants for different effectors of the Cys^{12} mutants ($S_{C12S}L_{wt}$ and $S_{C12A}L_{wt}$) and compare them with those of the wild-type in the oxidized state. There is a possibility that reduction can make the enzyme work more efficiently, for example, by increasing the affinity for the activator rather than increasing the V_{max} in the presence of activator. On the contrary, oxidation can decrease the affinity of the enzyme for the activator. This might be physiologically relevant since the enzyme is believed to operate in a highly inhibited environment (7). Under this circumstance, sensitivity to the activator is important for the function of the enzyme.

The physiological importance of the reductive activation phenomenon in the potato tuber enzyme is unclear. This issue is also complicated by the fact that the potato tuber is a nonphotosynthetic tissue and little information is available regarding redox changes inside the amyloplast. Nevertheless, since Cys^{12} and its surrounding sequence is conserved in the small subunit of ADPGlc PPases from all dicot plants sequenced to date, it will be interesting to test if a similar reductive activation mechanism also exists in photosynthetic tissues. Determination of the intermolecular disulfide bridge will also be important to understand the quaternary structure of ADPGlc PPase. It can provide very helpful information to solve the three-dimensional structure after getting the x-ray diffraction pattern of the enzyme, which is the focus of current research to understand the structure basis for the function of this enzyme.

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