Plant Physiol. (1995) 109: 245–251

Adenosine 5′-Diphosphate-Glucose Pyrophosphorylase from Potato Tuber[1]

Significance of the N Terminus of the Small Subunit for Catalytic Properties and Heat Stability

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cDNAs encoding the large subunit and a possibly truncated small subunit of the potato tuber (*Solanum tuberosum* L.) adenosine 5′-diphosphate-glucose pyrophosphorylase have been expressed in *Escherichia coli* (A.A. Iglesias, G.F. Barry, C. Meyer, L. Bloksberg, P.A. Nakata, T. Greene, M.J. Laughlin, T.W. Okita, G.M. Kishore, J. Preiss, J Biol Chem [1993] 268: 1081–1086). However, some properties of the transgenic enzyme were different from those reported for the enzyme from potato tuber. In this work, extension of the cDNA was performed to elongate the N terminus of the truncated small subunit by 10 amino acids. This extension is based on the almost complete conservation seen at the N-terminal sequence for the potato tuber and the spinach leaf small subunits. Expressing the extended cDNA in *E. coli* along with the large subunit cDNA yielded a transgenic heterotetrameric enzyme with similar properties to the purified potato tuber enzyme. It was also found that the extended small subunit expressed by itself exhibited high enzyme activity, with lower affinity for activator 3-phosphoglycerate and higher sensitivity toward inorganic phosphate inhibition. It is proposed that a major function of the large subunit of adenosine 5′-diphosphate-glucose pyrophosphorylases from higher plants is to modulate the regulatory properties of the native heterotetrameric enzyme, and the small subunit's major function is catalysis.

ADP-Glc PPase (ATP:α-Glc-1-P adenylyltransferase, EC 2.7.7.27) catalyzes the synthesis of ADP-Glc from Glc-1-P and ATP, releasing PPi as a product. This reaction is considered a prime, regulatory step in the synthesis of bacterial glycogen and of starch in plants (Preiss, 1984, 1988, 1991). The potato (*Solanum tuberosum* L.) tuber enzyme is a heterotetramer comprising two distinct subunits of 51 and 50 kD (Okita et al., 1990). Even though there is a little difference in molecular mass, we keep the nomenclature "large" for the 51-kD subunit and "small" for the 50-kD subunit, since they have homology to the large and small subunits, respectively, from all plants sources studied so far. The small subunit of higher plant ADP-Glc PPases is highly conserved, whereas the similarity among different large subunits is lower (Smith-White and Preiss, 1992).

The potato tuber enzyme ADP-Glc PPase is activated by 3-PGA and is inhibited by Pi, as are most higher plant, algal, and cyanobacterial ADP-Glc PPases (Preiss, 1982, 1988, 1991), and is heat stable in crude extracts, like many other ADP-Glc PPases from different sources (Sowokinos and Preiss, 1982). There has been no study of the protein structure of the potato tuber ADP-Glc PPase because of the difficulty of obtaining a highly pure and stable enzyme. Therefore, much effort has been directed at recombinant DNA techniques to obtain an enzyme expressed in *Escherichia coli*. Furthermore, the possibility to express the subunits separately would facilitate the study of the function of the large and small subunits. For that reason, it became important to have a system in which the recombinant ADP-Glc PPase behaves like the purified form from potato tuber.

The genes of both subunits of the ADP-Glc PPase were cloned (Nakata et al., 1991). However, the cloned small subunit lacked 10 amino acids of the N terminus by comparison with the mature spinach leaf small subunit, the only small subunit whose N-terminal sequence has been determined by protein sequencing (Morell et al., 1987) (Table I). Previous attempts to sequence the N terminus of the subunits of the purified enzyme from potato tuber failed because they were blocked. The amino acid sequence of the potato tuber small subunit is very similar to the spinach leaf small subunit at the N terminus (Morell et al., 1987; Nakata et al., 1991). It is believed, therefore, that the actual N-terminal sequence of the mature large and small subunits of potato tuber are very similar to those of the spinach leaf enzyme. For that reason, the cloned subunit lacking the 10 amino acids is defined in the present work as the "truncated" small subunit. Those cDNAs correspond-

[1] This work was supported in part by Department of Energy grants No. DE-FG02–93ER20121 (to J.P.) and DE-F6–0687ER136 (to T.W.O.). M.J.L. was supported in part by Biotechnology Training grant No. T32 GM08336.

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Abbreviations: $A_{0.5}$, $I_{0.5}$, $S_{0.5}$, concentrations of activator, inhibitor, and cofactor required for 50% of maximal activation, inhibition, and catalytic activity, respectively; ADP-Glc PPase, ADP-Glc pyrophosphorylase; LB, Luria broth; 3-PGA, 3-phosphoglycerate.

ing to the two subunits of the potato enzyme have been expressed together in an *E. coli* strain (AC70R1–504) deficient in endogenous ADP-Glc PPase activity to yield a functional enzyme (Iglesias et al., 1993). That recombinant ADP-Glc PPase resembled the purified enzyme from potato tuber, although it was not heat stable and the regulatory properties were slightly different. The recombinant enzyme had a different apparent affinity for 3-PGA and was much less sensitive to inhibition by Pi. One postulated explanation of these differences was that the 10 amino acids of the N terminus of the small subunit could be involved in the stability of the protein and the regulatory properties of the enzyme (Iglesias et al., 1993).

In this study, a new plasmid was constructed that contained a cDNA clone of the potato tuber small subunit. This clone encodes a polypeptide that has 10 amino acids more at the N terminus than the truncated small subunit. This expanded polypeptide is called the small subunit in this work and is now more similar to the mature spinach leaf ADP-Glc PPase at the N-terminal sequence. The resultant recombinant heterotetrameric enzyme comprising both the small subunit and large subunit is heat stable and is more sensitive to Pi inhibition, very similar to the enzyme purified from potato tuber. Of great significance is that the small subunit expressed by the new clone in the absence of the large subunit is also enzymatically active if high concentrations of 3-PGA are present in the reaction mixture. The large subunit alone had negligible catalytic activity.

MATERIALS AND METHODS

Reagents

[^{14}C]Glc-1-P and [^{32}P]PPi were purchased from Dupont. All other reagents were of the highest available commercial grade.

Plasmids

Plasmid pMON17336 is an expression vector that contains an insert encoding the large subunit of ADP-Glc PPase of potato (*Solanum tuberosum* L.) tuber. pMON17335 is another expression vector that contains an insert that encodes a truncated small subunit. The construction of both plasmids was described previously (Iglesias et al., 1993). Plasmid pMLaugh10 was a derivative from pMON17335 according to the following procedure. A complete copy of the small subunit was previously assembled, combining a cDNA clone and a portion of a genomic clone (Nakata et al., 1991, GenBank/EMBL accession No. X61186). The translation of the original cDNA clone started at NSQTCLDPDAS . . . (Anderson et al., 1991). The first amino acid (Asn) corresponds to amino acid 78 (fig. 2, Nakata et al., 1991). In the complete copy, a Met was engineered by PCR-based mutagenesis at residue 71 introducing an *Nco*I restriction site using the following oligonucleotide: 5′-GGGGTCGCCCATGGCTGTTTCTGATTCG-3′. Following PCR amplification, the putative full-length *Nco*I/*Kpn*I fragment of the small subunit was ligated to pMON17335 cut with *Nco*I/*Kpn*I. The *Kpn*I site is unique, as is *Nco*I, and is in the coding region (Iglesias et al., 1993). The nucleotide sequence of the clone was confirmed by sequencing. The new plasmid pMLaugh10 encodes a polypeptide corresponding to the putative potato mature small subunit (Table I). This polypeptide has 10 amino acids more than the polypeptide encoded by pMON17335. The other amino acids in the potato tuber small subunit, MIVSPKA, prior to what we consider the N terminus of the mature subunit, are believed to be part of the transit peptide portion of the small subunit. Even though the first 6 amino acids in the N terminus of the small subunit expressed by pMLaugh10 were elucidated by a genomic clone, the identity of this region with the purified spinach leaf small subunit is total (Table I).

Table I. *N-terminal amino acid sequences of the small subunit of ADP-Glc PPase from potato tuber and spinach leaf*

In this table, the position numbers are given according to those of Nakata et al. (1991) for the cDNA clone. pMON17335 and pMLaugh10 are plasmids described in "Materials and Methods." Underlined are the amino acids included in the newly designed clone of the small subunit of ADP-Glc PPase from potato tuber (pMLaugh10). Amino acid sequence of the pMLaugh10 expression product was deduced from the DNA sequence and determined as indicated in "Materials and Methods."

Source of ADP-Glc PPase	Amino Acid Sequence	Method	Reference
	7 8		
Potato tuber small subunit	0 0		
cDNA clone	. .MIVSPKAVSDSQNSQTCLDPDA. .	Deduced from DNA	Nakata et al. (1991)
pMON17335	MALDPDA. .	Deduced from DNA	Iglesias et al. (1993)
pMLaugh10	MAVSDSQNSQTCLDPDA. .	Deduced from DNA	This work
	AVSDSQNSQTXL.	Protein sequencing	This work
Spinach leaf small subunit purified from leaf	VSDSQNSQ.	Protein sequencing	Morell et al. (1987)
cDNA clone	VSDSQNSQTCLDPEA. .	Deduced from DNA	Smith-White et al. (1992)

Assay of ADP-Glc PPase

Pyrophosphorolysis Direction (Assay A)

Pyrophosphorolysis of ADP-Glc was followed by the formation of ATP from ^{32}PPi. The reaction mixture contained 20 μmol of Gly-Gly buffer (pH 8.0), 1.25 μmol of $MgCl_2$, 0.75 μmol of DTT, 2.5 μmol of NaF, 0.5 μmol of ADP-Glc, 0.38 μmol of ^{32}PPi (0.5–2.0 × 10^6 cpm/μmol), 50 μg of crystalline BSA, 1 μmol of 3-PGA, and the aliquot of enzyme in a volume of 0.25 mL. The reaction was started by the addition of enzyme and, after 10 min of incubation at 37°C, was terminated by the addition of 3 mL of cold 5% TCA. The [^{32}P]ATP formed was measured as previously described (Morell et al., 1987).

Synthesis Direction (Assay B)

Synthesis of ADP-Glc was followed by the formation of [^{14}C]ADP-Glc from [^{14}C]Glc-1-P. Reaction mixtures contained in 0.2 mL: 20 μmol of Hepes buffer (pH 8.0), 1 μmol of $MgCl_2$, 0.6 μmol of DTT, 0.1 μmol of [^{14}C]Glc-1-P (1.0 × 10^6 cpm/μmol), 0.3 μmol of ATP, 0.3 unit of inorganic pyrophosphatase, and 40 μg of crystalline BSA. 3-PGA was added when the enzyme was assayed in the presence of activator. Assays were initiated by addition of the enzyme. Reaction mixtures were incubated for 10 min at 37°C and terminated by heating in a boiling water bath for 1 min. [^{14}C]ADP-Glc was assayed as previously described (Ghosh and Preiss, 1966). Inhibition by Pi was measured by addition of potassium phosphate (pH 7.0). Kinetics parameters were determined in saturating conditions of substrates and cofactors (conditions mentioned above). If it is not indicated, the concentration of 3-PGA used in assay B was 3 mM. In both assays (A and B), 1 unit is defined as the amount of enzyme that produces 1 μmol of product in 1 min.

Protein Determination

Protein concentrations were determined by the method of Smith et al. (1985).

Molecular Mass Determination

The molecular mass of the native enzyme and the small subunit expressed in *Escherichia coli* was determined on 5 to 20% Suc density gradients according to the method of Martin and Ames (1961). The gradients were performed in 50 mM Hepes, pH 7.5, 2 mM EDTA.

Expression

E. coli strain AC70R1–504 containing plasmids pMON17336 and pMLaugh10 from a 15% glycerol stock stored at −80°C was inoculated in 5-mL LB liquid cultures containing 25 μg/mL kanamycin and 70 μg/mL spectinomycin and cultured overnight at 37°C. Then, 5 mL were transferred to 100 mL of fresh LB plus kanamycin and spectinomycin and cultured overnight at 37°C. Fifty milliliters of this culture were used to inoculate 1 L of LB plus spectinomycin and kanamycin in a 2.8-L Fernbach flask. This culture was incubated at room temperature until the A_{600} reached 1.2 to 1.4. At this time 5 μg/mL nalidixic acid and 10 μM isopropyl-β-D-thiogalactopyranoside were added to induce expression of the ADP-Glc PPase subunits. After 40 h of expression, cells were chilled on ice and harvested by centrifugation at 3000*g* for 5 min ("cell paste"). When the *E. coli* strain AC70R1–504 contained either plasmid pMLaugh10 or pMON17335, the procedure was the same except spectinomycin and nalidixic acid were omitted. When the only plasmid present in the *E. coli* strain was pMON17336, kanamycin and isopropyl-β-D-thiogalactopyranoside were omitted. With this protocol of expression, the specific activity of the recombinant enzyme (large plus small) was at least approximately 20-fold higher (1.4–2.0 units/mg) than the one previously reported (Iglesias et al., 1993).

Heat Treatment

Crude extracts, 0.2 mL, in a 1.5-mL Eppendorf tube were treated at the indicated temperature for 5 min and then cooled on ice and centrifuged at 14,000*g* for 10 min. Aliquots were withdrawn from the supernatant to assay activity in the pyrophosphorolysis direction. The 100% control was another aliquot of sample without treatment. As a purification step, 50 mL of crude extract in a 250-mL Erlenmeyer flask were heated in a water bath at 65°C with continuous agitation. The crude extract reached 65°C in approximately 2 min. Once at this temperature, the solution was kept for 5 min more in the bath before cooling quickly on an ice-water bath. After the sample was centrifuged at 12,000*g* for 15 min, the supernatant was kept at −80°C until further purification steps.

Purification of Recombinant Enzymes

Assay A was used to follow the activity during the purification. The recombinant enzyme containing the large subunit and the truncated small subunit was purified as previously described (Iglesias et al., 1993). The small subunit and the recombinant enzyme comprising the small subunit and the large subunit were purified as follows: Cell paste (approximately 10 g) was resuspended in 50 mL of buffer A (50 mM Hepes, pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 20% Suc) and disrupted by sonication as previously described (Iglesias et al., 1993). The crude extract was heat treated as indicated above. The additional steps were performed as indicated earlier (Sowokinos and Preiss, 1982) with the following changes. The buffers did not contain GSH and, in the purification of the small subunit, ammonium sulfate was used instead of potassium phosphate when chromatography on a C-3 hydrophobic column was performed. The final preparation of the small subunit had a specific activity of 20 units/mg protein and it was estimated to be approximately 50% pure by SDS-PAGE with a single contaminant of approximately 54 kD. The final preparation of the enzyme with both subunits had a specific activity of 39 units/mg and it was estimated to be 70 to 80% pure with only the same contaminant of approximately 54 kD. In western blots, the small subunit gave a strong reac-

tion with an antibody raised against spinach leaf ADP-Glc PPase (Morell et al., 1987). The large subunit gave a weak reaction, but the upper contaminant did not react. The mobility of the bands did not change during the purification process.

The N terminus of the purified small subunit was automatically sequenced after running 8 μg of protein in SDS-PAGE and blotting to a polyvinylidene difluoride membrane, staining with Coomassie blue R250, and cutting the band as described by LeGendre and Matsudaira (1989). The obtained sequence was: AVSDSQNSQT(X)L. This sequence confirms that the 10 extra amino acids are expressed (Table I). Only the first Met seems to be processed. Residue 11, which should be Cys, was not identified.

The purified, native enzyme and the purified, native small subunit enzyme had essentially the same molecular masses, 202 and 201 kD, respectively, as determined by Suc density gradient centrifugation (Martin and Ames, 1961). Since the large subunit is 51 kD and the small subunit is 50 kD in SDS-PAGE, the purified native enzyme is a heterotetramer and the purified small subunit is a homotetramer.

RESULTS

Activity of Different Subunits

As reported in a previous paper, the recombinant enzyme containing both a large and a truncated small subunit had activity in the crude extract (0.07 unit/mg); however, the activity of any of the subunits expressed alone was very low in the pyrophosphorolysis direction (assay A) (Iglesias et al., 1993). In this study, the new cDNA clone of the small subunit, pMLaugh10, when expressed alone, had a high specific activity in the crude extract (0.2 unit/mg). This activity was about 10-fold less than the activity obtained when both of the subunits were expressed together (specific activity of pMON17336 plus pMLaugh10, 1.8 units/mg). This allowed us to partially purify the small subunit. The activity of the large subunit expressed alone was very low in the conditions of the assay (<0.001 unit/mg) in the crude extract. The activity of the large subunit in the crude extract was not increased even if the concentrations of Mg^{2+}, ADP-Glc, and 3-PGA were increased to 10, 8, and 20 mM, respectively (<0.001 unit/mg).

Heat Stability

ADP-Glc PPases from different sources such as bacteria and plants have been able to retain the activity after a treatment between 60 and 70°C (Ozaki and Preiss, 1972; Morell et al., 1987; Iglesias et al., 1991). Heat treatment was used in the purification of the enzyme from potato tuber, but this was not possible with the recombinant enzyme with a truncated small subunit (Sowokinos and Preiss, 1982; Iglesias et al., 1993). It was therefore suggested that the small subunit may lack some amino acids at the N terminus that may be involved in maintaining heat stability (Iglesias et al., 1993). To test this hypothesis, the new ADP-Glc PPase having 10 more amino acids in the N terminus of the small subunit was expressed in *E. coli* and treated above 60°C as indicated in "Materials and Methods." The enzyme containing the truncated small subunit was not stable at 60°C (Table II) and was almost completely inactivated at 65°C as has been previously reported (Iglesias et al., 1993). Conversely, the enzyme that contained the complete small subunit was stable at 60 as well as 65°C. The latter enzyme after the purification was completely heat stable (100%) as long as the protein concentration had been kept above 1 mg/mL (data not shown). Not only did the expanded small subunit confer heat stability to the enzyme but also this subunit is stable per se because it remained fully active after 5 min at 65° (Table II). Since the only difference between both recombinant enzymes is the N terminus of the small subunit, we conclude that these amino acids, or some of them, are important to stabilize the protein structure. Furthermore, the extra amino acids abolished the difference in heat stability between the recombinant enzyme and the enzyme purified from potato tuber. For this reason, the N terminus encoded in the plasmid pMLaugh10 is a better candidate for the fully mature small subunit.

Table II. *Heat treatment of the expression products of different plasmids containing cDNA clones of potato tuber ADP-Glc PPase in E. coli strain AC70R1–504*

The expression of the plasmids was performed as indicated in "Materials and Methods" except the induction time was 3 h for pMON17336 + pMON17335 and pMON17336 + pMLaugh10 and 20 h for pMLaugh10. The specific activity (100%) was 0.04, 0.17, and 0.19 unit/mg in pMON17336 + pMON17335, pMON17336 + pMLaugh10, and pMLaugh10. The protein concentration was 1.9, 1.8, and 3.1 mg/mL in pMON17336 + pMON17335, pMON17336 + pMLaugh10, and pMLaugh10, respectively.

Transformant	Heat Treatment Temperature	Remaining Activity
	°C	%
pMON17336 + pMON17335 (large + truncated small subunits)	60	24
	65	1.4
pMON17336 + pMLaugh10 (large + small subunits)	60	89
	65	83
pMLaugh10 (small subunit)	60	98
	65	102

Pi Inhibition

Since the extent of inhibition by Pi depends on the concentration of activator, we studied the effect of Pi inhibition on the recombinant heterotetrameric enzyme at different concentrations of 3-PGA. The Pi $I_{0.5}$ of ADP-Glc synthesis increased from 40 to 630 μM when the concentration of 3-PGA was increased from 0 to 3 mM (Fig. 1A). It is interesting that the full-length small subunit alone was much more sensitive to Pi inhibition, even at higher concentrations of activator, than the heterotetrameric enzyme. When the concentration of 3-PGA was 3 mM, the small subunit was inhibited 50% with 80 μM phosphate. At the same concentration of 3-PGA, the $I_{0.5}$ for the recombinant heterotetrameric enzyme was 8-fold higher (Fig. 1A).

Activation by 3-PGA

The small subunit expressed alone had a high activity as long as the concentration of 3-PGA was at least 4 mM. The $A_{0.5}$ for the small subunit was 2.4 mM (Table III) in the synthesis direction and 2.5 mM in the pyrophosphorolysis direction. These values indicate that the purified homotetrameric small subunit has much lower affinity for the activator, since the $A_{0.5}$ for the recombinant heterotetrameric enzyme was 15-fold lower in the synthesis direction (Table III) and 96-fold lower in the pyrophosphorolysis direction (data not shown). Activation by 3-PGA and Pi inhibition were the main kinetics differences between the homotetrameric small subunit and the heterotetrameric ADP-Glc PPase. At saturating concentrations of 3-PGA (3 mM for the heterotetramer, 20 mM for the small subunit) $S_{0.5}$ for Mg^{2+} and K_m for ATP and Glc-1-P were 2.0, 0.120, and 0.040 mM for the heterotetramer and 2.2, 0.200, and 0.029 mM for the small subunit, respectively.

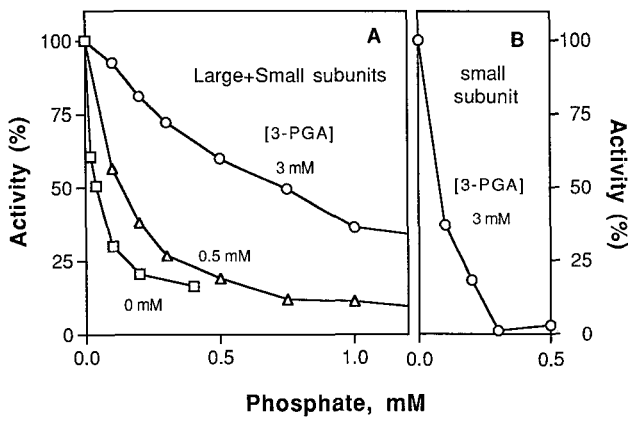

Figure 1. Inhibition by Pi of the small subunit and the heterotetrameric ADP-Glc PPase expressed in *E. coli*. The synthesis of ADP-Glc was assayed as indicated in "Materials and Methods" at different concentrations of 3-PGA and potassium phosphate. The activity was assayed on the purified expression products of plasmids pMON17336 plus pMLaugh10 (A) and pMLaugh10 (B). For the heterotetrameric enzyme (A), control activities (100%, in absence of Pi) were 27.3, 19.5, and 1.1 units/mg when the concentrations of 3-PGA were 3, 0.5, and 0 mM, respectively. For the small subunit (B), control activity was 7.5 units/mg. One unit is 1 μmol ADP-Glc synthesized min^{-1} mg^{-1} protein.

Table III. *Synthesis of ADP-Glc: activation by 3-PGA on different ADP-Glc PPases purified from different sources*

Enzyme	$A_{0.5}$
	μM
pMON17336 + pMON17335 (large + truncated small subunits) (Iglesias et al., 1993)[a]	57
Potato tuber, purified (Sowokinos and Preiss, 1982)	400
pMON17336 + pMON17335 (large + truncated small subunits)[a]	34
pMON17336 + pMLaugh10 (large + small subunits)[a]	160
pMLaugh10 (small subunit)[a]	2380

[a] Recombinant enzymes purified from *E. coli*.

Consistent with the idea that the heterotetrameric recombinant enzyme containing a fully mature small subunit is more similar to the purified enzyme from potato, we found that the $A_{0.5}$ for the former was almost 4.7-fold higher than the heterotetrameric recombinant enzyme with a 10-amino acid, truncated small subunit (Table III). That recombinant enzyme containing the truncated small subunit was more sensitive to activation by 3-PGA than the purified enzyme from potato, since $A_{0.5}$ values measured in synthesis direction were 0.057 and 0.400 mM, respectively (Sowokinos and Preiss, 1982; Iglesias et al., 1993). These results showed that the inclusion of the extra amino acids at the N terminus of the truncated small subunit overcame the differences between the recombinant and the purified enzyme from potato with respect to the allosteric regulation. Concurrently, there was no significant difference in sensivity to Pi inhibition between the recombinant enzyme (results mentioned above) and the purified enzyme from potato tuber (Sowokinos, 1981; Sowokinos and Preiss, 1982).

DISCUSSION

Expression and characterization of a potato tuber recombinant ADP-Glc PPase has been reported (Iglesias et al., 1993). However, it possessed slightly different kinetics properties and lacked the heat stability of the native tuber enzyme. Here we have obtained a recombinant enzyme with the more similar kinetics properties to the purified enzyme from potato. By inclusion of 10 amino acids in the N terminus of the previous small subunit (now called truncated small subunit based on the N-terminal sequence of the mature spinach leaf enzyme [Morell et al., 1987]), we could restore the characteristics absent in the recombinant enzyme previously published (Iglesias et al., 1993). In this study we show that the recombinant enzyme was as heat stable as the native enzyme. Furthermore, it had similar kinetics properties regarding Pi inhibition and activation by 3-PGA. The main difference is a slightly higher affinity toward Glc-1-P. The recombinant enzyme had a K_m of 40 μM, whereas the purified enzyme from potato had a K_m of 140 μM (Sowokinos and Preiss, 1982).

To support the idea that the heterotetrameric recombinant enzyme obtained in this work behaves like the native one, the rest of the kinetics parameters did not change significantly with the modification of the N terminus. The K_m for ATP (120 μM) and the $S_{0.5}$ for Mg^{2+} (2.0 mM) were similar to the enzyme purified from potato tuber and the recombinant enzyme with the truncated small subunit (Sowokinos and Preiss, 1982; Iglesias et al., 1993). In addition, the recombinant enzyme needed DTT to increase the activity between 2- and 4-fold, as did the native and the previous recombinant enzyme with the truncated small subunit (Sowokinos and Preiss, 1982; Iglesias et al., 1993). The recombinant enzyme is a heterotetramer similar to the enzyme from potato, since the molecular mass determined by Suc density gradients is about 200 kD. For all of these reasons, we conclude that the N terminus of the small subunit is involved in changes in the structure that alters the heat stability of the protein and its regulatory properties.

A very important point arose with the extension of the N terminus of the small subunit of the potato tuber ADP-Glc PPase. This subunit expressed alone is highly active (22 units/mg, a 50% partially purified protein) in the presence of relatively high concentrations of 3-PGA.The specific activity of the purified native potato tuber enzyme is 56.9 units/mg (Okita et al., 1990) and of the recombinant enzyme is 63.8 units/mg (Iglesias et al., 1993). This small subunit, in the absence of the large subunit, exists as a homotetramer, since the molecular mass was about 200 kD in nondenaturing conditions. This is in good agreement with previous studies in which it was shown that an ADP-Glc PPase from a mutant of Arabidopsis containing only the small subunit had activity at high concentrations of activator (Li Li and Preiss, 1992). The partially purified enzyme from the Arabidopsis mutant was also a tetramer. Because of the high enzyme activity seen with the small subunit, it is quite possible that it is the subunit primarily involved in catalysis. Because of its poor apparent affinity for the activator and higher sensitivity to Pi inhibition, we suggest that a major function of the large subunit is to modify the regulation of the small subunit. The large subunit alone had negligible catalytic activity; however, in the presence of the small subunit, it can increase the affinity for the activator and simultaneously decrease the Pi inhibition. It should be noted that recent studies indicate that the allosteric activator-binding sites are present in both the small and large subunits of the spinach leaf enzyme (Ball and Preiss, 1994). These sequences are highly conserved in the potato tuber subunits.

As indicated by Smith-White and Preiss (1992), the small subunit of higher plant ADP-Glc PPases is highly conserved, whereas there is great variation in similarity of the large subunit. It is quite possible that the differences seen in the large subunit allow differences in modulating sensitivity of the small subunit to allosteric activation and inhibition. Expression of various large subunits could differ during development or in their occurrences in different plants and different tissues (e.g. leaf, stem, guard cells, tuber, endosperm, root) and provide ADP-Glc PPases with differing sensitivities to regulation. The involvement of amino acid residues or domains in the catalysis and regulation of the enzyme can now be studied in a more facile manner with this new system of expression using chemical modification and via site-directed mutagenesis experiments.

ACKNOWLEDGMENT

We thank Dr. Mirta Sivak for critical review of the manuscript.

Received March 24, 1995; accepted June 6, 1995.

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