Expression of the Potato Tuber ADP-glucose Pyrophosphorylase in *Escherichia coli*

(Received for publication, June 29, 1992)

Alberto A. Iglesias‡, Gerard F. Barry‡, Christopher Meyer‡, Leonard Bloksberg‡, Paul A. Nakata§, Thomas Greene§, Mary J. Laughlin§**, Thomas W. Okita§, Ganesh M. Kishore§, and Jack Preiss††

From the ‡Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824, ¶Plant Sciences Technology, The Agricultural Group, The Monsanto Company, St. Louis, Missouri 63198, and the §Institute of Biological Chemistry, Washington State University, Pullman, Washington 99164

cDNA clones encoding the putative mature forms of the large and small subunits of the potato tuber ADP-glucose pyrophosphorylase have been expressed separately and together in an *Escherichia coli* B mutant deficient in ADP-glucose pyrophosphorylase activity. Expression of both subunits from compatible vectors resulted in restoration of ADP-glucose pyrophosphorylase activity. Maximal enzyme activity required both subunits. The expressed ADP-glucose pyrophosphorylase was purified and characterized. The recombinant enzyme exhibited catalytic and allosteric kinetic properties very similar to the enzyme purified from potato tuber. The expressed enzyme activity was neutralized by incubation with antibodies raised against potato tuber and spinach leaf ADP-glucose pyrophosphorylases but not with anti-*Escherichia coli* enzyme serum. 3-Phosphoglycerate was the most efficient activator and its effect was increased by dithiothreitol. In the ADP-glucose synthesis direction, 3-phosphoglycerate activated the recombinant enzyme nearly 100-fold in the presence of dithiothreitol, with an $A_{280}$ value of 57 $\mu$m. The recombinant ADP-glucose pyrophosphorylase was less sensitive to $P_i$ inhibition and more sensitive to heat denaturation than the potato tuber enzyme. Results suggest that bacterial expression of potato tuber cDNAs could be used to study the role and interaction of the subunits of the native ADP-glucose pyrophosphorylase.

ADP-glucose (ADP-Glc)$^1$ pyrophosphorylase (ATP:α-glucose-1-phosphate adenyltransferase, EC 2.7.7.27) plays a pivotal role in the biosynthesis of glycogen and starch in bacteria and plants, respectively (1, 2). This enzyme mediates the synthesis of ADP-Glc and $P_i$, from Glc-1-P and ATP, and the product, ADP-Glc, serves as the activated glucosyl donor in α-1,4-glucan synthesis. The catalytic activity of both the bacterial and plant enzymes are subject to allosteric regulation by small effector molecules, the levels of which vary during normal carbon metabolism in these organisms (1, 2). ADP-Glc pyrophosphorylase from enteric bacteria is activated by fructose-1,6-bisphosphate and inhibited by AMP (1). The enzyme found in the leaves of higher plants (2, 3), green algae (3, 4), cyanobacteria (5), and several non-chlorophyllous plant tissues (2, 3, 6-9) is allosterically activated by 3PGA and inhibited by $P_i$. Substantial evidence now exists indicating that, in the leaf as well as in algae, the ratio of 3PGA to $P_i$ within the chloroplast regulates starch synthesis by affecting ADP-Glc pyrophosphorylase activity (4, 10-12).

ADP-Glc pyrophosphorylase from all sources is found to be a tetrameric protein. However, the enzyme derived from enteric bacteria is homotetrameric (1, 2), whereas the enzyme from angiosperm plants is more complex in structure, being composed of two subunits that give rise to an $\alpha_2\beta_2$ heterotetrameric native enzyme (2, 13, 14). Since the bacterial and plant enzymes catalyze the same reactions, their structural dissimilarities may reflect their differences in allosteric specificity. A high structural homology between small subunits of higher plant ADP-Glc pyrophosphorylase has been reported (2, 15, 16), with analysis of cDNA clones corroborating these observations (16). The large subunits of plant enzyme were the most divergent and shared less sequence identity (14, 16). On the basis of this homology between them (15, 16), it has been speculated that the two plant subunits were originally derived from the same gene. During evolution there was gene duplication of the pyrophosphorylase gene followed by divergence of the genes to produce two polypeptides, both of which may be required for optimal activity (2, 3, 15, 16). This idea is reinforced by recent evidence showing that the cyanobacterial enzyme, which has the higher plant allosteric specificity, is a homotetramer like the bacterial enzyme (5, 17).

ADP-Glc pyrophosphorylase from potato tuber has been purified to homogeneity (14). The enzyme is composed of two different subunits of molecular mass 50 and 51 kDa (14). Kinetic studies showed that the potato tuber enzyme is regulated as a typical plant ADP-glucose pyrophosphorylase; thus, it is activated by 3PGA and inhibited by $P_i$ (7, 8). A major problem found for a complete characterization of this protein is that purification procedures results in low recoveries and the enzyme is quite unstable when isolated from potato (8, 14). Recently, cDNA clones to the small (18-20) and the large (20) subunits of the potato tuber ADP-Glc pyrophosphorylase have been isolated and may provide new tools for a complete characterization of the potato tuber enzyme. In this work, we report on the expression of the cDNA clones in an *Escherichia coli* B mutant deficient in the pyrophosphorylase activity. The purified recombinant protein showed properties similar to the potato tuber enzyme.
Experimental Procedures

Construction of pMON17335 and pMON17336

The compatible E. coli vectors for the expression of the mature forms of the potato tuber ADP-glucose pyrophosphorylase subunits were completed as follows. A complete copy of the small subunit cDNA was first assembled by combining a portion of the genomic clone and the almost complete cDNA clone (20). A methionine (and alanine) residue were then engineered before the leucine at residue 83 (20) by polymerase chain reaction-based mutagenesis using the following oligonucleotide: GAATTCCAGGGCCATGGCATTT-GACCCAGATGC. The C terminus of the coding region was engineered with the following oligonucleotide to introduce a Sac1 restriction site sequence: CCAAGTTAAAACGGAGCTCATCAGATGAT.

The reaction was started by the addition of 3'PPi, and after 10 min incubation at 37 °C it was terminated by the addition of 3 ml of cold 5% trichloroacetic acid. The [32P]ATP formed was assayed as described previously (13). A unit of ADP-Glc pyrophosphorylase activity is defined as that amount of enzyme catalyzing synthesis of 1 pmol of ATP/min under the reaction conditions described.

Assay A — Pyrophosphorylation of ADP-Glc was followed by the formation of ATP from [32P]PPi. The reaction mixture contained 20 μmol of glycyglycine buffer (pH 8.0), 1.25 μmol of MgCl₂, 0.75 μmol of DTT, 2.5 μmol of NaF, 0.5 μmol of ADP-Glc, 0.38 μmol of [32P]PPi, (1.0 to 6.0 × 10⁶ cpm/μmol), 50 μg of crystalline bovine serum albumin, 1 μmol of 3PGA, and enzyme in a final volume of 0.25 ml. The reaction was started by the addition of PpE₀ and after 10 min incubation at 37 °C it was terminated by the addition of 3 ml of cold 5% trichloroacetic acid. The [32P]ATP formed was measured as described previously (13). A unit of ADP-Glc pyrophosphorylase activity is defined as that amount of enzyme catalyzing synthesis of 1 pmol of ATP/min under the reaction conditions described.

Assay B — [m-14C]Gl-1-P was used to measure the synthesis of [14C]ADP-Glc. Standard reaction mixtures contained in 0.2 ml: 20 μmol of Hepes buffer (pH 8.0), 1 μmol of MgCl₂, 0.6 μmol of DTT, 0.1 μmol of [14C]Gl-1-P (1.0 × 10⁶ cpm/μmol), 0.3 μmol of ATP, 0.3 units of inorganic pyrophosphatase, and 40 μg of crystalline bovine serum albumin. 3PGA (0.5 μmol) was added when the enzyme was assayed in the presence of activator. Assays were initiated by addition of enzyme. Reaction mixtures were incubated for 10 min at 37 °C and terminated by heating in a boiling water bath for 30 s. [14C]ADP-Glc was assayed as previously described (23).

Kinetic Studies

$S_{0.5}$, $A_{0.5}$, and $I_{50}$ values, corresponding to the concentrations giving 50% maximal activity, activation, and inhibition, respectively, and Hill coefficients ($\eta_n$) were calculated from Hill plots (24). All kinetic parameters are the mean of at least two determinations and are reproducible to within at least ±10%.

Antibody Neutralization

Neutralization of the ADP-Glc pyrophosphorylase activity was performed basically as previously described (9). About 0.05 unit of the partially purified recombinant potato enzyme was diluted with 3 μmol of Hepes-NaOH, pH 7.0, containing 10 μg of bovine serum albumin, 1.25 μmol of P₀, 0.1 μmol of DTT, 5 mg of sucrose, and 50 μl of serum containing varying amounts of either anti-potato, anti-spinach leaf, or anti-E. coli ADP-Glc pyrophosphorylase immune serum diluted into the corresponding preimmune serum in a total volume of 0.1 ml. The mixture was incubated for 30 min at 30 °C and then for 2 h on ice prior to centrifugation for 5 min in Eppendorf microcentrifuge. Enzyme activity in the supernatant was measured by using assay A.

Protein Determination

Protein concentration was measured after Smith et al. (25) using Pierce prepared bicinchoninic acid (BCA) reagent and bovine serum albumin as the standard.

PAGE and Western Blotting

PAGE for the native enzyme was performed in the Ornstein-Davis system (26) using 7.5% and 5% acrylamide gels for resolving and stacking, respectively. SDS-PAGE was performed in 9% gels according to Laemmli (27). After electrophoresis, proteins on the gel were transferred onto nitrocellulose membranes as described by Burnette (28). Following electroblotting, nitrocellulose membranes were treated with mouse anti-potato tuber or affinity-purified rabbit anti-spinach leaf ADP-Glc pyrophosphorylase antisera and the antigen–antibody complex was visualized as previously described (5).

Expression of ADP-Glc Pyrophosphorylase in E. coli

E. coli strain AC70R1-504 containing plasmids pMON17335 and pMON17336 were inoculated from 15% stocks stored at ~70 °C onto Luria broth (LB) plates containing 15 μg/ml kanamycin and 50 μg/ml spectinomycin and cultured overnight at 37 °C. Single colonies

**Fig. 1.** Potato tuber ADP-Glc pyrophosphorylase expression vectors. A, plasmid pMON17335 is a derivative of pACYC177 (21) and expresses the mature form of the small subunit from the tac promoter (40) and the phage T7 gene10 leader (GIOl) translation enhancer (41). B, the plasmid pMON17336 was used to introduce a methionine in the putative mature region of the large subunit: GGATCACCAGGGCCATGGCATTT-GACCCAGATGC. This coding sequence was then cloned as an NcoI-Sac1 fragment into a pACYC-based vector to form pMON17335 (Fig. 1). The following oligonucleotide was used to introduce a methionine in the putative mature region of the large subunit: GAATTCCAGGGCCATGGCATTT-GACCCAGATGC. This coding sequence was then cloned as an NcoI-Sac1 fragment into a pACYC-based vector to form pMON17335 (Fig. 1). The following oligonucleotide was used to introduce a methionine in the putative mature region of the large subunit: GGATCACCAGGGCCATGGCATTT-GACCCAGATGC. This coding sequence was then cloned as an NcoI-Sac1 fragment into a pACYC-based vector to form pMON17335 (Fig. 1). The following oligonucleotide was used to introduce a methionine in the putative mature region of the large subunit: GGATCACCAGGGCCATGGCATTT-GACCCAGATGC. This coding sequence was then cloned as an NcoI-Sac1 fragment into a pACYC-based vector to form pMON17335 (Fig. 1).
were inoculated into 4-ml LB cultures containing kanamycin and spectinomycin as above for 12 h before subculturing 750 μl into 750 ml of fresh LB plus kanamycin and spectinomycin in 2.8-liter Fernbach flasks for overnight incubation at 37 °C. Late log phase cultures of fresh LB plus kanamycin and spectinomycin were inoculated into 4-ml LB cultures containing kanamycin and spectinomycin in 2.8-liter Fernbach flasks for overnight incubation at 37 °C. Late log phase cultures were used to inoculate M5CA medium (42 mM Na2HP04, 22 mM KH2PO4, 8.6 mM NaCl, 19 mM NH4Cl, 100 μM CaCl2, 5 mM MgSO4, 0.2% glucose, 0.2% casamino acids) at a cell density of 1.5 liter of 100 liters of M5CA in a Braun model 120 fermenter. Cells were grown for 7 h to OD600 = 0.738 prior to adding 100 μM isopropyl-β-D-thiogalactopyranoside and 5 μg/ml nalidixic acid to induce expression of the ADP-Glc pyrophosphorylase subunits. After 3 h of expression, cells were chilled to 10 °C by adding ice and harvested in a refrigerated Sharples centrifuge. Cell paste was kept overnight at -20 °C before use.

### Purification of the Recombinant ADP-Glc Pyrophosphorylase

All steps were carried out at 0-4 °C. Assay A was used to monitor enzyme activity throughout the purification. Cell paste was resuspended in extraction buffer (about 5 ml of buffer/g of cells) containing 50 mM glycylglycine (pH 7.5), 5 mM MgCl2, 1 mM EDTA, 5 mM DTT, and 20% sucrose. The suspension was disrupted by sonic oscillation in a Heat Systems Ultrasonic sonicator model W-220F and then centrifuged at 12,000 × g for 15 min. The pellet was washed once in additional buffer (about half the volume of the original homogenate) and centrifuged. The supernatants were combined (crude extract) and applied to a DEAE-Sepharose fast-Flow column (2.25 × 37 cm) that had been equilibrated with the extraction buffer supplemented with 5 mM potassium phosphate (pH 7.5). After washing with 5-bed volumes of the above buffer, the enzyme was eluted with a linear gradient consisting of 4-bed volumes of the extraction buffer in the mixing chamber and 4-bed volumes of 50 mM potassium phosphate (pH 6.0), containing 2 mM DTT and 0.4 M KCl in the reservoir chamber. The fractions containing ADP-Glc pyrophosphorylase activity were pooled and the protein precipitated by addition of crystalline ammonium sulfate up to 60% saturation.

The precipitate was collected by centrifugation, dissolved in a small volume of medium (buffer A) containing 20 mM Bis-Tris-propane buffer (pH 7.0), 5 mM potassium phosphate, 5 mM MgCl2, 1 mM EDTA, 10% (w/v) sucrose, and 2 mM DTT, and then desalted by passing through Econo-Pac 10DG columns (Bio-Gel P-6 gel desalting gel from Bio-Rad) equilibrated with buffer A. This sample (28 ml) was applied to a Mono Q HR10/10 column equilibrated with buffer A. The column was washed with 40 ml of buffer A and eluted with a linear KCl gradient (100 ml, 0-0.5 M) in buffer A. Fractions of 5 ml were collected, and those containing activity were pooled and then concentrated to 10 ml in an Amicon concentrator fitted with a PM 30 membrane. The concentrated enzyme fraction was diluted with equal volume of 2 M potassium phosphate (pH 7.0) and then applied to an aminopropyl-agarose column (C2 column, 1 × 12 cm) previously equilibrated with 1 M potassium phosphate buffer (pH 7.0) containing 2 mM DTT. The enzyme was absorbed, and the column was successively washed with 10-bed volumes of each of the following P; buffers (pH 7.0): 1, 0.75, 0.5, 0.20, and 0.05 M. The enzyme was eluted with 0.75 M P buffer. Fractions containing ADP-Glc pyrophosphorylase activity were pooled and concentrated by Amicon ultrafiltration with a PM 30 membrane and then dialyzed for 12 h against 1 liter of the buffer used for Mono Q chromatography (buffer A). This fraction was stored at -80 °C, and the enzyme, under these conditions, was fully active for at least 3 months.

## RESULTS

Plastid-targeted proteins are typically processed during import into the plastid by removal of a N-terminal transit peptide. The N terminus of the plastid-localized subunits has only been determined for the spinach leaf ADP-glucose pyrophosphorylase (13). By comparison of the aligned protein sequences of the ADP-glucose pyrophosphorylases from plant and bacterial sources (20), a putative mature N-terminal region has been determined for the subunits of the potato tuber enzyme. This assignment is also in agreement with our current understanding of the structural features of transit peptides. Following the addition of an initiator methionine, the putative mature subunit coding regions have been expressed separately and together (from compatible expression vectors) in E. coli. Crude extracts of E. coli AC70R1-504 cells transformed with the different plasmids were analyzed for ADP-Glc pyrophosphorylase activity. As shown in Table I, expression of the small (plasmid pMON17335) or large (plasmid pMON17336) subunit separately resulted in extracts containing low ADP-Glc pyrophosphorylase activity. Enzyme activity could be partially reconstituted by mixing equal amounts of each of the above extracts (Table I). E. coli cells transformed with both pMON17335 + 17336 yielded extracts containing the highest ADP-Glc pyrophosphorylase activity (Table I).

The recombinant enzyme was found to exhibit a heat stability different from that of the native potato ADP-Glc pyrophosphorylase. The enzyme purified from potato remains fully active after 5 min incubation at 70 °C, and this treatment was utilized as a purification step in potato crude extracts (8, 14). Under the same conditions, the recombinant potato ADP-Glc pyrophosphorylase is almost completely inactivated.

In order to characterize the recombinant ADP-Glc pyrophosphorylase in greater detail, it was purified from E. coli AC70R1-504 cells transformed with pMON17335+17336. In crude extracts the recombinant enzyme accounted for approximately 0.13% of the total soluble protein of the cells, based on the specific activity of the pure native potato enzyme determined previously (14). Table II summarizes a typical purification of ADP-Glc pyrophosphorylase from 132 g (100 liter culture) of E. coli transformed cells. The purification procedure resulted in a 952-fold purified enzyme with a specific activity of 63.8 units/mg and 50% recovery.

Native PAGE of the purified recombinant enzyme showed a major protein band (data not shown). In SDS-PAGE, two major protein bands of molecular masses about 50 and 80 kDa were observed. Immunoblot analysis of SDS-PAGE showed that only the broad band of molecular mass 50-52 kDa was recognized by anti-potato tuber and anti-spinach leaf ADP-Glc pyrophosphorylase immune sera, thus suggesting that it corresponds to the two subunits of the potato recombinant enzyme. Results suggest that the preparation is about 50% pure and it contains a major contaminant, which co-migrates with the recombinant enzyme in native PAGE. Although the recombinant enzyme was partially purified, its specific activity (63.8 units/mg) was slightly higher than that reported for the enzyme purified from potato tuber (56.9 units/mg) for an enzyme nearly 50% pure; see Ref. 14).

Kinetic and regulatory properties of the purified recombinant ADP-Glc pyrophosphorylase were determined in both pyrophosphorolysis and ADP-Glc synthesis directions. The results were compared with those previously reported for the potato tuber enzyme (7, 8). Table III shows the effect of different metabolites on the physiological synthetic activity of the enzyme. 3PGA behaved as the most potent activator by increasing the enzyme activity over 50-fold. Fructose-1,6-

### Table I

**Expression of plasmids containing cDNA clones for each subunit (separately and together) of potato tuber ADP-Glc pyrophosphorylase in E. coli strain AC70R1-504**

| Transformant          | ADP-Glc pyrophosphorylase activity* |
|-----------------------|-------------------------------------|
|                       | milligrams/mg                       |
| 1. None               | <0.3                                 |
| 2. pMON17335 (small subunit) | 1.0                               |
| 3. pMON17336 (large subunit)  | 1.1                               |
| 4. Combined extracts (2 + 3)         | 10.2                              |
| 5. pMON17335 + 17336 (both subunits) | 70.0                              |

*Activity was assayed in the pyrophosphorolysis direction (assay A).
Potato Tuber ADP-glucose Pyrophosphorylase in E. coli

TABLE II
Purification of potato tuber ADP-Glc pyrophosphorylase expressed in E. coli AC70R1-304

| Step                  | Volume (ml) | Protein (mg) | Activity (units) | Specific activity (units/mg) | Purification (fold) | Yield (%) |
|-----------------------|-------------|--------------|-----------------|-----------------------------|---------------------|-----------|
| Crude extract         | 875         | 5650         | 376             | 0.867                       | 1                   | 100       |
| DEAE-Sepharose        | 375         | 550          | 312             | 0.57                        | 8.5                 | 83        |
| Ammonium sulfate      | 28          | 420          | 313             | 0.75                        | 11.2                | 83        |
| Mono Q                | 50          | 76           | 290             | 3.83                        | 57.2                | 77        |
| C₉ column             | 15          | 5            | 188             | 63.8                        | 952                 | 50        |

TABLE III
Effect of different metabolites on the activity (ADP-Glc synthesis direction) of recombinant potato tuber ADP-Glc pyrophosphorylase

| Effector             | ADP-Glc formed (nmol/10 min) | Relative activity |
|----------------------|------------------------------|-------------------|
| None                 | 0.6                          | 1.0               |
| Glucose 1,6-bisphosphate | 0.8                             | 1.3               |
| Fructose 6-phosphate | 0.9                          | 1.5               |
| Fructose 1,6-bisphosphate | 1.9                             | 3.3               |
| Pyruvate             | 0.9                          | 1.5               |
| Phosphoenolpyruvate  | 2.3                          | 4.0               |
| 3-phosphoglycerate   | 20.7                         | 36                |
| 2-Phosphoglycerate   | 1.0                          | 1.7               |
| 2,3-Bisphosphoglycerate | 1.0                              | 1.7               |
| Pi                   | 0.5                          | 0.9               |

![Fig. 3](image.png)

**Fig. 3.** Activation by 3PGA of recombinant potato tuber ADP-Glc pyrophosphorylase. The ADP-Glc synthesis assay method is described under "Experimental Procedures," except that the specified amounts of 3PGA were added without further additions (C) or in the presence of 4 mM DTT (O).

bialized the recombinant enzyme activity. The amounts of antiserum causing 50% inhibition were about 300 and 225 μl/unit of enzyme for anti-potato tuber and anti-spinach leaf serum, respectively. Interestingly, antibody for the E. coli ADP-Glc pyrophosphorylase (up to 1700 μl/unit) caused no inhibition of the recombinant enzyme activity (Fig. 2).

The activation of the recombinant ADP-Glc pyrophosphorylase by 3PGA was studied in more detail. In the pyrophosphorylase direction, 3PGA exhibited a hyperbolic pattern with a calculated Aₐ₅ value of 45 μM and a 3-fold maximal activation. DTT (4 mM) slightly decreased the Aₐ₅ for 3PGA to 34 μM without significantly affecting the degree of the activation. Fig. 3 shows that the recombinant enzyme was activated 3-fold by 3PGA in the ADP-Glc synthesis direction. Activation by different 3PGA concentrations followed hyperbolic kinetics with an Aₐ₅ value of 160 μM. The presence of 4 mM DTT increased the affinity of the recombinant enzyme toward the activator (Aₐ₅ = 57 μM) and enhanced the maximal activation by nearly 2-fold (Fig. 3) as observed with the potato tuber enzyme (8).

Effective inhibition by P_i of the ADP-Glc synthesis activity of the recombinant enzyme required of the presence of 3PGA in the assay medium and maximal inhibition values of only 20% were obtained with 4 mM P_i in the absence of 3PGA. The effect of P_i on the rate of ADP-Glc synthesis at three different 3PGA concentrations is shown in Fig. 4. Fifty percent inhibition occurred with P_i concentrations of 83, 320, and 680 μM in the presence of 10, 125, and 250 μM 3PGA, respectively. In all cases, the inhibition curve was sigmoidal, with n_i values of 2.6 (10 μM 3PGA), 1.6 (125 μM 3PGA), and 3.0 (250 μM 3PGA). When assayed in the pyrophosphorylase direction, the enzyme was not inhibited by P_i, even in the presence of 3PGA.

To characterize the recombinant ADP-Glc pyrophosphorylase in greater detail, the kinetic parameters of the enzyme
in the absence and in the presence of 2.5 mM 3PGA were determined. The results are compared with the values previously reported for the potato tuber enzyme in Table IV. As shown, 3PGA decreased the \( S_{0.5} \) value for ADP-Glc, PP, and Glc-1-P of the recombinant enzyme, with a higher increase in affinity observed for Glc-1-P. The activator also changed the sigmoidal pattern exhibited by ADP-Glc to a hyperbolic saturation curve for this substrate. These results are, as a whole, in good agreement with the kinetic parameters previously reported for the potato tuber ADP-Glc pyrophosphorylase (7, 8). The main difference found for the recombinant enzyme is that it exhibited a higher apparent affinity toward Glc-1-P (Table IV). \( S_{0.5} \) values for Mg\(^{2+}\) were also determined for the potato enzyme expressed in E. coli. Under all the experimental conditions, the divalent cation saturation curve was sigmoidal. Table IV shows that in the ADP-Glc synthesis direction, 3PGA increased the affinity of the recombinant enzyme for Mg\(^{2+}\) by almost 2-fold.

### Table IV

**Kinetic parameters for substrates of partially purified ADP-Glc pyrophosphorylase expressed in E. coli ACT0R1-504 and comparison with the potato tuber enzyme**

| Substrate | Recombinant enzyme | Potato tuber enzyme* |
|-----------|--------------------|----------------------|
|           | \( S_{0.5} \) | \( n_{H} \) | \( S_{0.5} \) | \( n_{H} \) |
| Pyrophosphorolysis direction | | | | |
| ADP-Glc | 0.54 | 1.9 | 0.83 | Sig |
| ADP-Glc (+ 3PGA)* | 0.26 | 1.1 | 0.24 | Hyp |
| PP | 0.22 | 1.0 | 0.23 | Hyp |
| PP1 (+ 3PGA) | 0.13 | 1.1 | 0.10 | Hyp |
| Mg\(^{2+}\) | 2.10 | 2.5 | ND | |
| Mg\(^{2+}\) (+ 3PGA) | 1.95 | 2.4 | ND | |
| ATP (+ 3PGA) | 0.22 | 1.2 | ND | |
| ATP | 0.24 | 1.0 | 0.19 | Hyp |
| Glc-1-P | 0.26 | 1.2 | ND | |
| Glc-1-P (+ 3PGA) | 0.087 | 1.0 | 0.14 | Hyp |
| Mg\(^{2+}\) | 3.10 | 2.3 | ND | |
| Mg\(^{2+}\) (+ 3PGA) | 1.51 | 3.0 | ND | |
| ADP-Glc synthesis direction | | | | |
| 3PGA | 0.087 | 1.0 | 0.14 | Hyp |
| 3PGA | 1.95 | 2.4 | ND | |
| 3PGA | 3.10 | 2.3 | ND | |
| 3PGA | 1.51 | 3.0 | ND | |

*Kinetic data for the potato tuber enzyme, in the pyrophosphorylase and ADP-Glc synthesis direction, were obtained from Refs. 7 and 8, respectively.

*3PGA concentration was 2.5 mM.*

DISCUSSION

We have constructed different plasmids containing cDNA encoding the putative mature primary sequence of the large and/or small subunit of the potato tuber ADP-Glc pyrophosphorylase. These plasmids were used to transform cells of E. coli B, strain ACT0R1-504, a glycogen-less mutant deficient in ADP-Glc pyrophosphorylase activity (30). Only low levels of enzyme activity were obtained when either subunit was expressed alone. High enzyme activity levels were achieved when both expression vectors were simultaneously used to transform the E. coli cells. To the best of our knowledge, this is the first time that two dissimilar eukaryotic genes have been expressed together in E. coli rendering a functional enzyme.

The recombinant enzyme was purified and characterized and its properties compared with the potato tuber ADP-Glc pyrophosphorylase. The recombinant enzyme expressed in E. coli possesses immunologic, kinetic, and allosteric regulatory properties similar to the potato tuber enzyme. Thus, (i) the recombinant enzyme was recognized by polyclonal antibodies raised against plant ADP-Glc pyrophosphorylases but not by the anti-E. coli enzyme serum; (ii) kinetic parameters for Glc-1-P, ATP, ADP-Glc and PP, determined in this study were very similar to those reported for the enzyme purified from potato tuber (7, 8); and (iii) 3PGA was the most important activator of the recombinant enzyme, the activation kinetics being in agreement with those found in the potato tuber pyrophosphorylase (7, 8).

It is worth considering two main differences found between the recombinant enzyme and the native ADP-Glc pyrophosphorylases. One difference results from the low heat stability exhibited by the recombinant protein. The second is the lower inhibitory effect of P\(_{i}\) on the enzyme expressed in E. coli. It is tempting to speculate that both differences could be due to the same cause. Heat treatment at 60–70 °C has been successfully used as a purification step for ADP-Glc pyrophosphorylase from bacteria and plant (5, 8, 13, 31). In many cases, it is necessary to add P\(_{i}\) (20–30 mM) to the medium prior to the heat treatment in order to adequately protect the enzyme from heat denaturation. Thus, the lower affinity of the recombinant ADP-Glc pyrophosphorylase for P\(_{i}\) could result in a reduced binding of this compound to the protein, enabling the anion to effectively protect the enzyme against thermal inactivation. Interestingly, P\(_{i}\) alone gave very low inhibition of the recombinant enzyme activity, suggesting that it binds poorly to the protein. The inhibitory effect of P\(_{i}\) observed when 3PGA was also present, and the fact that higher 3PGA concentration resulted in a higher \( I_{0.5} \) for P\(_{i}\), suggest some kind of interaction between both compounds in their binding to the allosteric sites of the enzyme. Thus, higher concentrations of 3PGA can desensitize the enzyme to P\(_{i}\) inhibition. Taking into account that the small subunit cDNA in pMON17335 lacks sequences coding for about 10 residues of the N terminus or the possible presence of mutations produced during the cloning and isolation of the large and small subunit cDNA plasmids, it is quite possible that the expressed protein is lacking part of the P\(_{i}\)-binding site.

In the pyrophosphorolysis direction, the recombinant ADP-Glc pyrophosphorylase was activated 3-fold by 3PGA with an \( A_{0.5} \) value nearly 40 \( \mu \)M. This \( A_{0.5} \) value is higher than that reported for the potato tuber enzyme (\( A_{0.5}=5 \mu \)M; see Ref. 7). On the contrary, the kinetic parameters for 3PGA activation in the ADP-Glc synthesis direction showed a higher affinity of the recombinant enzyme toward the activator (\( A_{0.5}=160 \) and 57 \( \mu \)M in the absence and in the presence of DTT, respectively) as compared with the potato tuber enzyme (\( A_{0.5}=
400 μM; see Ref. 8). These discrepancies could be attributed to many factors. One possibility is that the different purification degrees of the enzyme utilized in each study is affecting the apparent affinity observed for 3PGA. In this way, the significant differences in the conditions during extraction and purification could amplify this effect. It is possible that the presence of polyphenol oxidase, which is prevalent in potato tuber, and/or proteases modify the activator site during the purification procedure whereas in E. coli these activities may not be present. The differences found in 3PGA activation could also be explained by the fact that the expression of the truncated small subunit cDNA clone results in an enzyme with a slightly modified regulatory site.

Despite the importance of starch synthesis in reserve tissues such as potato tuber, a complete characterization of the enzymes involved in the metabolism is far from complete. Concerning ADP-Glc pyrophosphorylase from potato tuber, the enzyme has been purified and partially characterized (7, 8, 14) and the heterotetrameric structure of the enzyme was unequivocally demonstrated only recently (14, 20). As indicated above, purification of the potato tuber ADP-Glc pyrophosphorylase is quite difficult because of the low stability of the protein resulting in final recoveries of activity lower than 10% (8, 14). Moreover, the catalytic efficiency of the enzyme purified from potato tuber seems to be affected by the instability conditions during purification, since its specific activity was found to be at least 2-fold lower than those reported for the enzyme from other sources (5, 13, 14). A main cause for this instability seems to be the presence of proteases and oxidases (e.g. polyphenol oxidase) in extracts from the plant tissue (14). The results showed herein on the expression of the potato tuber enzyme are promising because of the potential to obtain relatively high amounts of the enzyme in a medium that seems to be more adequate for the protein stability.

Several studies have shown the involvement of amino acid side chains in catalysis and regulation of ADP-Glc pyrophosphorylase from bacteria and plants (32–39). These studies were never performed with the potato tuber enzyme, mainly because of the difficulties in enzyme purification described above. Purification of the enzyme from transformed E. coli cells provides sufficient protein to identify residues using group-specific chemical modifiers. Moreover, the expression system should be an useful tool to perform site-directed mutagenesis to further characterize the role of certain amino acids in the protein. These studies to elucidate structure to function relationships in the recombinant enzyme are the focus of our current efforts.

Acknowledgments—We thank Peter Olins, Cathy Devine, and Shaukat Rangwala of Monsanto Corporate Research for kindly supplying the plasmids used in the construction of the ADP-glucose pyrophosphorylase expression vectors.

REFERENCES

1. Preiss, J., and Romeo, T. (1989) Adv. Microb. Physiol. 30,183-238
2. Preiss, J. (1991) Oxford. Plant. Mol. Cell Biol. 7, 59-114
3. Preiss, J. (1988) in The Biochemistry of Plants (Preiss, J., ed) pp. 181-254, Vol. 14, Academic Press, San Diego
4. Ball, S., Marianne, T., Dürick, L., Frenoy, M., Delrue, B., and Decq, A. (1991) Planta (Heidelb.) 185, 17-26
5. Iglesias, A. A., Kakefuda, G., and Preiss, J. (1991) Plant Physiol. 97, 1189-1196
6. Dickinson, D., and Preiss, J. (1969) Arch. Biochem. Biophys. 130, 119-128
7. Sowokinos, J. R. (1981) Plant Physiol. 68, 924-929
8. Sowokinos, J. R., and Preiss, J. (1982) Plant Physiol. 69, 1459-1466
9. Plaxton, W. C., and Preiss, J. (1987) Plant Physiol. 85, 105-112
10. Petersson, G., and Ryde Petersson, U. (1989) Eur. J. Biochem. 179, 169-172
11. Neuhaus, H. B., Kruckenberg, A. L., Feil, R., and Stitt, M. (1989) Planta (Heidelb.) 179, 110-122
12. Neuhaus, H. E., and Stitt, M. (1990) Planta (Heidelb.) 182, 445-454
13. Morel, M. K., Bloom, M., Knowles, V., and Preiss, J. (1987) Plant Physiol. 85, 182-187
14. Okita, T. W., Nakata, P. A., Anderson, J. M., Sowokinos, J., Morell, M., and Preiss, J. (1990) Plant Physiol. 83, 785-790
15. Preiss, J., Falke, K., Smith-White, B., Iglesias, A., Kakefuda, G., and Li, L. (1991) Bioclin. Soc. Trans. 19, 529-547
16. Smith-White, B., and Preiss, J. (1991) J. Mol. Evol. 34, 449-464
17. Kakefuda, G., Chary, Y.-Y., Iglesias, A. A., McIntosh, L., and Preiss, J. (1992) Plant Physiol. 99, 359-361
18. Muller-Korber, B. T., Kossmann, J., Haushofer, L. C., Willmitzer, L., and Sonnewald, U. (1990) Mol. Gen. Genet. 224, 136-146
19. unialdin, J., and Berlin, A. (1991) Plant Mol. Biol. 16, 349-359
20. Nakata, P. A., Greene, T. W., Anderson, J. M., Smith-White, B. J., Okita, T. W., and Preiss, J. (1991) Plant Mol. Biol. 17, 1089-1095
21. Chang, A. C. Y., and Cohen, S. N. (1978) J. Bacteriol. 134, 1141-1156
22. Soberon, A., Covarrubias, L., and Bollivar, F. (1980) Gene (Amst.) 9, 287-300
23. Ghosh, H. P., and Preiss, J. (1966) J. Biol. Chem. 241, 4491-4504
24. Dixon, M., and Webb, E. C. (eds) (1979) The Enzymes, 3rd Ed., pp. 332-487, Academic Press, New York
25. Smith, P. K., Krohn, R. I., Hermansson, G. T., Mallia, A. K., Gartner, P., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Kenk, D. C. (1985) Anal. Biochem. 140, 76-85
26. Gabriel, O. (1971) Methods Enzymol. 22, 565-578
27. Laemmli, U. K. (1970) Nature 227, 680-685
28. Burnette, W. N. (1981) Anal. Biochem. 112, 195-203
29. Keegstra, K., Olsen, L. J., and Theg, S. M. (1989) Annu. Rev. Plant Physiol. 40, 471-501
30. Carlson, C. A., Parsons, T. F., and Preiss, J. (1976) J. Biol. Chem. 251, 7800-7892
31. Ozaki, H., and Preiss, J. (1972) Methods Enzymol. 23, 405-413
32. Haugten, T., Ibaque, A., and Preiss, J. (1976) Biochem. Biophys. Res. Commun. 69, 348-353
33. Parsons, T. F., and Preiss, J. (1978) J. Biol. Chem. 253, 6197-6202
34. Parsons, T. F., and Preiss, J. (1978) J. Biol. Chem. 253, 7638-7645
35. Carlson, C. A., and Preiss, J. (1982) Biochemistry 21, 1929-1934
36. Moorell, M., Bloom, M., and Preiss, J. (1988) J. Biol. Chem. 263, 633-637
37. Lee, Y. M., Mukherjee, S., and Preiss, J. (1986) Arch. Biochem. Biophys. 244, 383-395
38. Iglesias, A. A., Kakefuda, G., and Preiss, J. (1992) J. Protein Chem. 11, 119-128
39. Ball, R., and Preiss, J. (1992) J. Protein Chem. 11, 231-238
40. DeBoer, H. A., Constock, L. J., and Vasser, M. (1985) Proc. Natl. Acad. Sci. U. S. A. 80, 21-25
41. Olins, P. O., and Kangwala, S. H. (1989) J. Biol. Chem. 264, 16973-16975
42. Fling, M., Kopf, J., and Richards, C. (1985) Nucleic Acids Res. 13, 7095-7106