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SUBSTRATE BINDING MUTANTS OF THE HIGHER PLANT ADP-GLUCOSE PYROPHOSPHORYLASE*

MARY J. LAUGHLIN,† JASON W. PAYNE‡ and THOMAS W. OKITA‡§

† Genetics and Cell Biology, ‡ Institute of Biological Chemistry, Washington State University, Pullman, Washington 99164, U.S.A.

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Key Word Index—ADP-glucose pyrophosphorylase; mutagenesis; starch; allosteric regulation; catalysis.

Abstract—To explore the structure-function relationships of the heterotetrameric higher plant ADP-glucose pyrophosphorylase, composed of a pair of large and small subunits, the small subunit cDNA was subjected to chemical mutagenesis and then co-expressed with the wild-type large subunit cDNA. Mutants were selected for their inability to complement a defective bacterial ADP-glucose pyrophosphorylase gene and, in turn, to accumulate glycogen as viewed by iodine staining of the cells. Based on these initial analyses, we subsequently identified four distinct classes of mutations which were glycogen-deficient but exhibited enzyme activity levels comparable to the normal recombinant enzyme under saturating reaction conditions. Three classes, each a product of single amino acid substitution, showed altered kinetic constants for substrates. Substitution of Asp252 to Asn conferred the enzyme lower affinity for glucose-1-phosphate, replacement of Asp121 to Asn resulted in an enzyme less responsive to both glucose-1-phosphate and ATP, while the Ala106 to Thr substituted enzyme contains altered sensitivity primarily to ATP. The fourth class, a Pro43 to Ser substitution, resulted in an enzyme with decreased sensitivity (8-fold) to the activator 3-PGA. Overall, the results of this study suggests that the two subunit types do not have identical roles in enzyme function and that the small subunit plays a more dominant role in catalysis than the large subunit.

INTRODUCTION

ADPglucose (ADP-Glc) pyrophosphorylase controls the initial step in α-glucan and starch synthesis in bacteria and plants [Glucose-1-phosphate + ATP → ADP-glucose + PPi] reviewed in [1–3]. Both the bacterial and plant enzymes are allosterically regulated by small molecules whose nature reflects the dominant carbon assimilatory pathways observed in these organisms. The enzyme from enteric bacteria is activated by fructose 1,6-bisphosphate and inhibited by AMP while the plant enzyme, with few exceptions, is activated by 3-phosphoglycerate (3-PGA) and inhibited by ortho-phosphate (Pi). Unlike the homotetrameric structure of the bacterial enzyme which is encoded by a single gene, the plant ADP-Glc pyrophosphorylase is a heterotetramer composed of a pair of large and small subunits encoded by distinct genes [3].

Although the small and large subunit share considerable structural identity (>50%) at the primary sequence level [4], available evidence suggests that these subunits play distinct roles in enzyme function. Under certain conditions, the small subunit is capable of forming a homotetramic enzyme [5]. In contrast, no activity is detected when the large subunit is expressed by itself. The capacity of the small subunit to self-assemble into a homotetrameric enzyme is only evident in the absence of the large subunit. The homotetrameric small subunit enzyme displays altered allosteric regulation as compared to the wild-type heterotetrameric enzyme. It is less sensitive to the allosteric activator 3-PGA ($A_{0.5}$ = 2.4 mM) than the wild-type enzyme ($A_{0.5}$ = 0.16 mM) and more sensitive to Pi with a Ki 8-fold lower than wild type.

In vivo evidence for a catalytic role of the small subunit is provided by an *Arabidopsis thaliana* mutant (*adg*2) which lacks an expressed large subunit but contains an active small subunit and has enzyme characteristics similar to the recombinant homotetrameric enzyme [6]. Like the recombinant homotetramer, the *adg*2 mutant requires increased 3-PGA concentration for maximum enzyme activity and is

* Dedicated to Professor Clarence 'Bud' Ryan on the occasion of his 65th birthday.

§ Author to whom correspondence should be addressed.

more sensitive to Pi inhibition. Additional support for the catalytic role of the small subunit comes from primary sequence homology comparisons between leaf, endosperm and tuber forms of the small subunit. Sequence comparisons show that the small subunit has been subjected to greater selection pressure than the large [7]. In potato in particular, the same small subunit gene is expressed in all tissues examined while multiple forms of the large subunit exist that are expressed in specific tissues [8–10]. Overall, the ability of the small subunit to form an active homotetramer when expressed without the large subunit [5], the conservation of primary sequence in the small subunit [4, 7], and the universal tissue expression of the small subunit found in potato [9, 11] indicates that the small subunit may play more of a catalytic function than the large subunit. In turn, the role of the large subunit is to modulate the catalytic activity of the small subunit by increasing its sensitivity to the activator 3-PGA, and conversely, lowering its sensitivity to the inhibitor Pi.

To explore the structure-function relationships of ADP-Glc pyrophosphorylase, we have capitalized on the ability of the co-expressed small and large subunits to form functional enzyme and complement a mutation in *glgC* (the structural gene for the bacterial host ADP-glc pyrophosphorylase) in *Escherichia coli*. Complementation restores glycogen accumulation, a biochemical process easily screened by iodine staining. Using this system we generated mutants in the large subunit that were less sensitive to allosteric activation by 3-PGA [12, 13]. In this study, the function of the small subunit was explored by random mutagenesis of the small subunit cDNA. Unlike the large subunit group I and IV mutants which are defective mainly in allosteric regulation, comparable type mutants of the small subunit, with one exception, are defective in their affinities for the substrates glucose-1-phosphate or ATP. The isolation of these mutants corroborates the hypothesis that the small subunit's role is more of the catalytic subunit while the role of the large subunit is to modulate this catalysis by altering sensitivity to the allosteric effectors.

DISCUSSION

ADP-Glc pyrophosphorylase catalyzes the first committed step in α-glycogen synthesis in bacteria and starch synthesis in plants [1–3]. Due to its pivotal role in starch synthesis and recent interest in modifying this enzyme for biotechnological uses to increase starch production, this study was performed to initiate examination of the interaction of the large and small subunits of this heterotetramic protein. Specifically, the role of the small subunit in enzyme catalysis and allosteric regulation was explored by a random mutagenesis approach using the chemical mutagen, hydroxylamine, to introduce defined C to T transitions. By first eliminating mutant lines which contained deficient small subunit levels caused by nonsense mutations as well as those effecting transcription and plasmid copy number, we were able to categorize the remaining mutant lines into six groups depending on their capacity to accumulate little or no glycogen but possessing significant levels of measurable catalytic activity under excess substrate and activator conditions.

Of particular interest in this study were mutants in Groups I and II which were unable to synthesize detectable levels of glycogen as viewed by I_2 staining but contain close to wild-type levels of enzyme activity. Of the glycogen deficient mutants containing single point mutations isolated with wild-type antigen levels and high levels of enzymatic activity, multiple lines of three of the substitutions were isolated. Specifically, two independent lines contained an Ala106Thr replacement, three independent lines contained an Asp121Asn exchange, two independent lines contained an Asp252Asn change while only one line contained a Pro43Ser substitution. The identified amino acid residues are completely conserved in both the large and small subunits of all known plant ADP-glucose pyrophosphorylase enzymes.

Much of the work on identification of substrate and allosteric binding sites in ADP-Glc pyrophosphorylase has been done on the homotetrameric *E. coli* enzyme. Mutation analysis as well as chemical modification studies have identified residues in the *E. coli* enzyme which lie at or near the substrate and allosteric sites. Specifically, two substrate binding sites have been identified in *E. coli*: the ATP and Glucose-1-phosphate binding sites. Tyr114 has been identified to be located at or near the ATP binding site in *E. coli* [3]. Chemical modification studies of the *E. coli* enzyme have shown Tyr114 to be involved in binding the adenine protein of ADP-glucose and the *E. coli* inhibitor AMP as well as ATP [1]. Two of the residue changes identified with this study in the heterotetrameric potato tuber enzyme, Ala106Thr and Asp121Asn, are within this region identified as the ATP binding site in the *E. coli* enzyme (Fig. 3). These substitution resulted in enzymes with altered affinity for the substrate ATP. The Asp121Asn mutation results in an enzyme with a 3-fold reduction in affinity for ATP and a 10-fold decrease in affinity for glucose-1-phosphate. The Ala106Thr substitution has a more complex affect on enzyme activity by altering not only the enzymatic interaction with ATP (8-fold), but also its interaction with the allosteric activator 3-PGA (2.8-fold), and the cofactor Mg^{2+} (3-fold). This Ala106Thr mutation introduces an amino acid that is both larger in size and contains a polar hydroxyl group, suggesting disruption of the ATP binding site. Assessment of the maximum velocity under saturating conditions and estimated purity for the Ala106Thr enzyme supports the view that the Ala106Thr mutation causes a significant alteration in enzyme structure which alters both catalytic and allosteric regulatory properties. Replacement of Tyr114 with Phe in the *E. coli* enzyme resulted in the formation of

an enzyme which also exhibited lower affinity to ATP (10-fold), the allosteric activator 3-PGA (26-fold) and the cofactor Mg^{2+} (13-fold) [21]. It is interesting that independently derived substitutions in bacteria and, with this study, in plants have identified this region around residue Tyr114 as being involved in forming the ATP binding pocket.

Residues located near or at the glucose-1-phosphate substrate binding sites have also been identified. The *E. coli* Lys195 has been identified at or near the glucose-1-phosphate binding site [22]. Site-directed mutagenesis of the potato small subunit Lys188 to glutamate, equivalent to the *E. coli* ADP-Glc pyrophosphorylase K195, increased the K_m for glucose-1-P from 80 μM to over 45 mM [3]. Using a random mutagenesis approach to isolate mutant enzymes, kinetic analysis of the substituted enzymes in this study reveals the major affect of the substitutions Asp252Asn and Asp121Asn to be a lower affinity for the substrate glucose-1-phosphate. Substituting Asp with Asn does not affect the amino acid size but results in the loss of charge, possibly altering the enzyme's affinity for substrates by disrupting hydrogen bonds and the conformation of the substrate binding site. The Asp121Asn substitution, which decreases the enzyme's affinity for glucose-1-phosphate 10-fold but also ATP and Mg^{2+} 3-fold, may disrupt both substrate binding sites, although the formation of the glucose-1-phosphate binding pocket is altered to a stronger extent than the ATP substrate binding site. A major structural change was detected in the Asp121Asn substituted enzyme by assessing maximum velocity under saturated conditions and taking into account the purity of the enzyme. Using this method, the catalytic activity of the Asp121Asn substituted enzyme was approximately 2-fold higher than wild-type. Thus the Asp121Asn substituted enzyme increases the overall catalytic activity of the enzyme but at the expense of decreased affinity for substrates.

Previous studies have identified putative regions in ADP-Glc pyrophosphorylase important for allosteric regulation in both the bacteria and plant enzymes. In higher plants, chemical modification of spinach leaf ADP-Glc pyrophosphorylase using pyridoxal-P, an analogue of the allosteric activator 3-PGA, has identified a single labeled peptide in the small subunit at the extreme carboxy-terminus of the enzyme [23]. In the spinach leaf large subunit, three distinct labeled peptides have been identified including a peptide at the same aligned carboxy-terminal site as identified in the small subunit [24]. In contrast, in the complementary study to this report, an allosteric mutant of potato ADP-Glc pyrophosphorylase has recently been obtained [12]. The single amino acid substitution responsible for the decreased sensitivity to 3-PGA was located at the amino terminal Pro52 in the large subunit. In our current study, the same analogous residue in the small subunit was independently identified as effecting sensitivity to the allosteric activator 3-PGA (Fig. 3). However, each subunits' control over allosteric regulation may not be to the same extent. When Pro52 was substituted with Leu in the large subunit, there was a 45-fold decrease in the enzyme's affinity for 3-PGA, while in the small subunit the similarly aligned Pro to Ser substitution resulted in an 8-fold decrease. Accessing the maximum velocity of the small subunit Pro43Ser substituted enzyme shows the enzyme exhibited approximately normal catalytic activity levels consistent with no gross changes in the overall confirmation of this substituted enzyme. The Pro43Ser replacement likely disrupts the structure of the allosteric binding pocket by removing the highly conserved Pro. Thus the small subunit participates to some extent in allosteric regulation of this heterotetrameric enzyme although to a lesser extent than the large subunit.

To determine if the identified shift in kinetic parameters could account for the observed *in vivo* phenotype, levels of the substrates and effectors in *E. coli* were estimated from the literature. Since *in vivo* levels of substrates and effectors are dependent upon growth conditions and the stage of growth at harvest, the published concentration of substrates and effectors in *E. coli* can only be estimates for our system. Mg^{2+} is estimated at 114 mM [25] and should not be limiting to the substituted enzymes. ATP and 3-PGA are estimated at 3 mM and 0.75 mM, respectively [26, 27] 3-PGA would certainly be limiting to the Pro43Ser substituted enzyme. 3-PGA alone or the combined 3-PGA/ATP affect could be limiting to the Ala106Thr substitution, the only substrate mutant enzyme with an altered $A_{0.5}$ [26]. Glucose-6-phosphate levels are estimated at 1.1 mM [26, 28]. At equilibrium, phosphoglucomutase equilibrates the concentration of glucose-6-phosphate and glucose-1-phosphate in the ratio 94:6 [29]. Glucose-1-phosphate may thus be estimated at 70 μM. Therefore glucose-1-phosphate levels would be extremely limiting to the enzymes substituted with Asp112Asn and Asp252Asn which would account for the decreased enzyme activity and resulting glycogen deficient phenotype. For confirmation, glucose-1-phosphate would need to be quantitated in the present growth media and conditions. Increased affinity for phosphate could also account for the observed phenotype, however the mutant enzymes effected in sensitivity to substrates exhibited up to a 2-fold decrease in their sensitivity to inhibitor.

An interesting observation from these random mutagenesis studies is that no Group I or Group IV enzymes with altered affinity for substrates have yet to be isolated when the large subunit was mutated [12, 13] supporting the catalytic role of the small subunit. In addition, when the large subunit was mutagenized, 29% of the mutants isolated belonged to Group VI (low glycogen levels with low *in vitro* enzymatic activity). In contrast, no Group VI mutants were isolated when the small subunit was mutagenized. This suggests that mutation of the small subunit to such an

extent that low levels of glycogen were produced *in vivo* requires medium to high levels of enzyme activity, while mutations in the large subunit decrease but do not eliminate enzymatic function *in vivo*. Thus although there is evidence for allosteric control by the small subunit, its main role appears to be as the major controlling factor in enzyme catalysis.

The identification of substrate mutants in the small subunit supports the catalytic role of the small subunit. Conservation of the identified amino acids illustrates the importance of these residues for enzyme function. Three of the amino acid residues identified, Pro43, Ala106 and Asp252, are completely conserved in bacteria, cyanobacteria and plants. Asp121 is also highly conserved, deviating only in *Bacillus*. In addition, in three of the mutants the mutagen used in this study introduced conservative amino acid changes into the mutant enzymes (Asp to Asn and Ala to Thr) and yet produced identifiable phenotypic changes. Site-directed mutagenesis with less conservative amino acid changes of the sites identified in this study may be able to further elucidate the structure-function of the small subunit. Ongoing work with this system should help clarify the roles of both subunits.

EXPERIMENTAL

Reagents. [^{14}C]Glucose-1-phosphate was purchased from American Radiolabeled Chemicals. [^{32}P]PPi was purchased from DuPont. All other compounds were of the highest available commercial grade.

Mutagenesis and characterization of mutants. The vector pML10 [5], containing the potato small subunit cDNA, was mutagenized with 0.8 M hydroxylamine as described previously [12]. A time course of exposure of pML10 to the mutagen was performed and the mutagenized plasmid was transformed into the *glgC*- and hence glycogen deficient *E. coli* strain AC70R1-504 containing the large subunit of potato tuber ADP-Glc pyrophosphorylase. Transformants were grown on LB with selection and replica plated onto 2% enriched media [14]. Screening for glycogen accumulation was performed using I_2 vapour. Initially over 100 mutants with altered glycogen production were characterized (intermediate staining and null-staining colonies when exposed to I_2 vapour). Protein extracts of the glycogen deficient mutants were prepared from cells grown on 2% enriched plates with selection. Cells were collected from the plates and resuspended in lysis buffer containing 20% sucrose, 50 mM Hepes pH 8.0, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulphonyl fluoride, 0.5 μg ml^{-1} pepstatin, and 0.5 μg ml^{-1} leupeptin. Cells were lysed by 4 cycles of freezing in liquid N_2 and thawing at 37°. Cell lysates were clarified by centrifugation and supernatants were used for further analysis. Small subunit antigen levels were assessed by enzyme-linked immunosorbent assay (ELISA) using rabbit polyclonal antibodies specific to the small subunit. Only 25% of the glycogen deficient mutants contained greater than 90% of the wild-type small subunit level and were further analysed. Mutants were quantitated for *in vitro* enzymatic activity with assay A (see below) and grouped according to *in vivo* glycogen levels and *in vitro* enzymatic activity (Table 1). In order to specifically isolate Group I mutants (null-staining mutants with wild-type enzyme activity levels), over 4000 additional transformants were screened by I_2 vapour resulting in the isolation of 119 null-staining mutants. Of these glycogen-minus mutants, only 11 contained wild-type small subunit antigen levels as assessed by ELISA and medium to high levels of enzyme activity as determined by assay A (see below). For DNA sequence analysis, these 11 mutants were cured of the large subunit plasmid by selection on kanamycin. Sequencing was performed using Sequenase as recommended by the manufacturer (United States Biochemical).

Assay of ADP-Glc pyrophosphorylase. The activity of ADP-Glc pyrophosphorylase was determined in both the pyrophosphorylase (assay A) or the synthesis (assay B) direction. Assay A: Pyrophosphorylase activity of ADP-Glc pyrophosphorylase was followed by the formation of [^{32}P]ATP from [^{32}P]PPi. The enzyme was assayed as previously described except the reaction was conducted in the presence of 20 mM 3-PGA [15]. A unit of ADP-Glc pyrophosphorylase activity is defined as the amount of enzyme which catalyzed the synthesis of 1 μmol of ATP min^{-1} under the reaction conditions described.

Assay B: Synthesis activity of ADP-Glc pyrophosphorylase was followed by the formation of [^{14}C]ADPglucose from [^{14}C]glucose-1-phosphate as described with some modification [12]. The reaction mixt. contained 100 mM Hepes pH 8.0, 5 mM $MgCl_2$, 3 mM DTT, 0.5 mM glucose-1-phosphate, 0.05 μCi

Table 1. Initial isolation and grouping of mutants which exhibit wild-type levels of small subunit antigen

Mutant group	Number of mutants	Glycogen status	Enzymatic activity relative to wild-type
I	2	Glycogen deficient	70–100%
II	2	Glycogen deficient	30–69%
III	5	Glycogen deficient	0–29%
IV	17	Low glycogen	70–100%
V	4	Low glycogen	30–69%
VI	0	Low glycogen	0–29%

[^{14}C]glucose-1-phosphate, 1.5 mM ATP, 0.3 U/reaction inorganic pyrophosphatase, 0.4 mg ml^{-1} BSA, and 3 mM 3-PGA in 200 μl. Reactions were incubated at 37° for 10 min and then terminated by boiling for 2 min. Calf intestinal alkaline phosphatase (1.5 U/reaction in 50 μl of 1X CIAP buffer (New England Biolabs) was added to each reaction and incubated at 37° an additional 60 min. 50 μl of reaction was spotted onto DEAE 81 paper, dried and counted. For inhibition by phosphate, potassium phosphate pH 7.0 was used. Under satd conditions, the reaction mixt. contained 15 mM $MgCl_2$, 4 mM glucose-1-phosphate, 0.4 μCi [^{14}C]glucose-1-phosphate, and 1.5 mM K^+ATP in place of the standard levels. Protein levels were determined by Bradford analysis using bovine serum albumin as the standard [16].

Enzyme purification. Crude protein extracts were prepd as described [5]. To prevent degradation of the enzyme during purification, a protease cocktail containing 1 μg ml^{-1} of pepstatin, leupeptin, chymostatin, aprotinin, benzamidine, and 1 mM phenylmethylsulphonyl fluoride was used. The crude protein extract was pptd with a 30–55% differential ammonium sulphate pptn and the pellet stored at −80° until further analysis. The pellet was resuspended in a minimal amount of lysis buffer (50 mM Hepes pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 20% sucrose), incubated at 60° for 5 min, clarified by centrifugation, and desalted over a Sephadex G25 column. The protein was then loaded onto a MemSep 1010 DEAE column (Millipore) equilibrated in buffer A (5 mM potassium phosphate pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 50 mM glyclglycine pH 7.5 and 20% sucrose) and eluted with a 0–400 mM KCl gradient in buffer B (50 mM potassium phosphate pH 6.0, 5 mM $MgCl_2$, 1 mM EDTA, 400 mM KCl, 20% sucrose). Active enzyme frs were pooled and concd using Centricell (Polysciences) centrifuge filters, aliquoted and stored at −80°. Purity and integrity of the partially purified enzymes was determined by analysis on 10% SDS polyacrylamide gels followed by Coomassie Brilliant Blue staining and immunoblot analysis with antibodies specific for ADP-Glc pyrophosphorylase large and small subunits. Percent purity was estimated from the SDS polyacrylamide gels stained with Coomassie Brilliant Blue using Biorad Molecular Analyst software program.

Kinetic Studies. K_m and $A_{0.5}$ values, corresponding to the levels required for 50% maximal activity or activation were calculated using Lineweaver-Burk plots determined by the computer program Enzymekinetics (Trinity Software). The K_m values for the cofactor Mg^{2+} was determined using non-linear regression plots determined as above. $I_{0.5}$ corresponding to the level required for 50% inhibition was calcd from the inhibition curve at the specified allosteric activator level using Sigmaplot. Maximum velocity was calculated under saturated conditions using Lineweaver–Burk plots determined by the computer program Enzymekinetics. All kinetic parameters are the result of at least two determinations.

Glycogen quantitation. Glycogen was quantitated utilizing a glucose-6-dehydrogenase coupled assay kit from Boehringer Mannheim. Cells were grown in 2% glucose enriched media with selection overnight, harvested, resuspended in H_2O and lysed by boiling for 3 min. After centrifugation, 500 mg of the ppt. was solubilized in 2 ml dimethylsulphoxide and 0.5 ml HCl at 60° for 30 min. Following solubilization, the sample was brought to pH 4 with NaOH in citrate buffer (0.112 mol l^{-1} pH 4.0) to a total vol. of 10 ml. Starch was hydrolysed to D-glucose by amyloglucosidase. The D-glucose formed was quantitated spectrophotometrically by measuring the formation of NADPH using a coupled enzyme assay system containing hexokinase and glucose-6-dehydrogenase.

RESULTS

Isolation of mutants in Groups I to VI

In order to explore the functional roles of the large and small subunits of potato ADP-Glc pyrophosphorylase, a co-expression system in *E. coli* has been developed [17]. Co-expression of the heterotetramer potato ADP-Glc pyrophosphorylase cDNAs on two expression vectors in a *glgC*-strain permits the study of the effects of mutations in one subunit on the allosteric or catalytic properties of the resulting heterotetrameric enzyme. Using this system, a significant number of mutants were obtained which were classified into 6 groups. Groups I, II and II were unable to complement a mutation in the *E. coli glgC* and contained normal, intermediate and low levels of enzyme activity, respectively, when assayed under very high saturating substrate and activator conditions [12]. Several of the group I, II and IV mutations have been characterized and found to exhibit defects in sensitivity to allosteric activation to 3-PGA [12, 13].

In this study, the role of the small subunit on regulation and function of the heterotetrameric enzyme was investigated using hydroxylamine-mediated mutagenesis of the small subunit cDNA. The hydroxylamine-treated DNA was transformed into an *E. coli glgC*-strain containing the large subunit cDNA plasmid. Mutation frequency was determined as a percentage of transformants which lacked the ability to efficiently restore glycogen production. In order to isolate single amino acid substitutions, transformation frequency was compared to mutagenesis frequency and the 20 hr mutagenesis time point (21% mutation frequency) was chosen to use in mutant isolation (see Fig. 1). In addition to null-staining colonies, these mutations included intermediate I_2 staining colonies which could be distinguished from the wild-type.

Since the entire small subunit plasmid was mutagenized, the apparent lack of complementation exhibited by the null-staining colonies may be due to

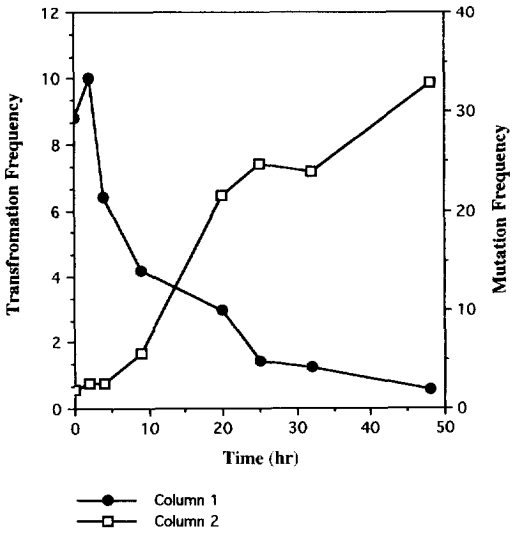

Fig. 1. Random mutagenesis of ADP-Glc pyrophosphorylase small subunit cDNA. Transformation frequencies and mutation frequencies for the mutated ADP-glucose pyrophosphorylase small subunit cDNA when expressed in *glgC- E. coli* strain containing the wild-type large subunit.

mutations in regions that cause deficient transcription or translation of ADP-Glc pyrophosphorylase. To eliminate these types of mutations, crude enzyme extracts from the null-staining and intermediate I_2 staining mutants were analysed by enzyme-linked immunosorbent assay specific for the small subunit. Only 25% of these mutants contained wild-type antigenic levels and were further evaluated for ADP-Glc pyrophosphorylase activity in the presence of saturating levels of ADPglucose (1 mM) and the activator 3-PGA (20 mM) to identify substrate binding and allosteric mutants. These mutants which expressed normal small subunit antigen levels were categorized into six groups depending on their level of glycogen accumulation (intermediate or null-staining) and enzyme activity, ranging from low (0–29% of wild-type), medium (30–69% of wild-type), to high pyrophosphorylase activity (70–100% of wild-type) (Table 1).

In this study, we were specifically interested in glycogen null mutants which did not stain when exposed to I_2 vapours. This mutant type occurs at a 3- to 7-fold lower frequency than the intermediate I_2 staining mutants. To this end, over 4000 additional transformants were screened by I_2 vapour resulting in the isolation of 119 null-staining mutants. These mutants were evaluated for small subunit antigen levels and enzymatic activity. The majority of these glycogen-deficient mutants lacked wild-type levels of small subunit antigen or enzymatic activity. However, members of Groups I and II exhibited significant enzymatic activity *in vitro* when assayed in the presence of excess substrates or alosteric activator.

The small subunit cDNA from the 11 glycogen-minus mutants (groups I and II) were sequenced to identify the molecular basis for the change in enzyme function. Several of the mutant lines were due to gross DNA segmental mutations which caused pronounced changes in the primary sequence. Eight mutants contained single point mutations which resolved into four classes, all except one with multiple members. Two mutant lines contained an Ala106Thr replacement, three mutants contained an Asp121Asn substitution, two mutants contained an Asp252Asn exchange while one mutant contained a Pro43Ser replacement (Table 2).

Three classes of the partially purified mutant enzymes contain altered sensitivity to substrates. The enzymes from one member of each of the four mutant classes were partially purified and kinetic analysis performed. Partial purification of the wild-type enzyme resulted in a 25-fold purification which is estimated to be 48% pure by SDS-PAGE (see Table 2 and Fig. 2). The mutant enzymes were partially purified with the same protocol resulting in approximately slightly less pure enzymes (see Table 2). SDS-PAGE followed by immunoblot analysis with antibody specific for ADP-Glc pyrophosphorylase small or large subunit showed no apparent degradation of the enzyme occurred during purification (Fig. 2). Prevention of degradation was essential since work on the barley, maize, and potato ADP-Glc pyrophosphorylase has shown that significant degradation of the protein during purification altered the kinetic regulatory properties of the enzyme (18, 19).

Kinetic analysis in the synthesis direction revealed

Table 2. Specific activity and glycogen levels of ADP-glucose pyrophosphorylase. Comparison of wild-type with substrate mutants isolated *in vivo*

	Amino acid change	Crude protein extract specific activity (μ mole min^{-1} mg^{-1})	Glycogen (mg starch g^{-1} wet weight)	Partially purified maximum velocity (μ mole min^{-1})*	% Purity of the partially purified enzymes†
Wild-type	—	0.059 (100%)	86.7 (100%)	23.3	48
Mutant 715	D252N	0.046 (78%)	5.5 (6%)	17.5	41
Mutant 772	A106T	0.031 (53%)	0.8 (1%)	10.1	42
Mutant 814	D121N	0.058 (98%)	1.8 (2%)	40.0	37
Mutant 947	P43S	0.060 (101%)	0.5 (1%)	18.9	47

* Mutants assayed under saturating conditions.

† Estimated as described in Experimental.

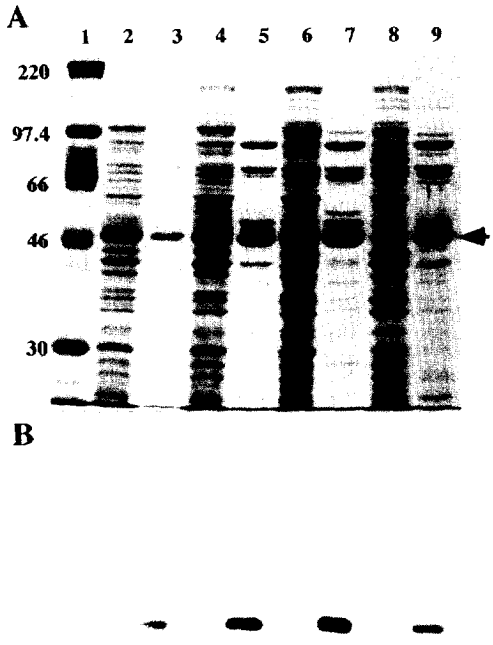

Fig. 2. SDS-PAGE and Immunoblot analysis of the mutants expressing enzyme with altered affinity for substrate(s). SDS-PAGE stained with Coomassie Brilliant Blue (A) and immunoblot using antibody specific for the small subunit (B) of wild-type ADP-glucose pyrophosphorylase and the identified mutants contain enzymes with altered affinity for substrate(s). Lanes 2,4,6 and 8 show crude protein extracts and lanes 3,5,7 and 9 contain the partially purified enzymes. Sample order is wild-type followed by the substituted enzymes Asp252Asn, Ala106Thr and Asp121Asn. For the SDS-PAGE shown in (A), crude extract lanes contain 30 μg total protein, wild-type partially purified lane contains 3 μg, and the mutant partially purified lanes contain 10 μg total protein. For the immunoblot shown in (B), sample order is the same as in (A) with all lanes containing 1 μg total protein.

three classes of mutant enzymes were effected by altered sensitivity to substrates (Table 3). Enzyme with the small subunit Asp252Asn substitution has a 6-fold lower affinity for the substrate glucose-1-phosphate (K_m = 1.21 mM) than wild-type (K_m = 0.19 mM). The enzyme binding constants for the substrate ATP and the allosteric activator 3-PGA were unaltered from the wild-type enzyme (Table 3). In addition, there was a 2-fold decrease in the enzyme's affinity for the cofactor Mg^{2+} (K_m = 4.5 mM) from wild-type (K_m = 2.1 mM).

Enzyme containing the Asp121Asn substitution displays normal allosteric activation by 3-PGA and inhibition by Pi. However this enzyme shows a significant 10-fold lower affinity for the substrate glucose-1-phosphate (K_m = 1.97 mM) than wild-type (K_m = 0.19 mM). There is also a 3-fold increase in the enzyme's binding constants for ATP (K_m = 0.52 mM) when compared to wild-type (K_m = 0.18 mM) and a 3-fold increase in the enzyme's binding constant for Mg^{2+}

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                S
32...RLYPLTKKRAKPAVPLGANY...51
41...KLFPLTSRTATPAVPVGGCY...60
32...RLKDLTNKRAKPAVHFGGKF...51

          T              N
100...FVEVLAAQQSPENPD..WFQGTADAVRQYL...127
109...FVEVLAATQTPGEAGKKWFQGTADAVRKFI...138
 98...FVDLLPAQQRMK..GENQYRGTADAVTQNL...125

190...RIIEFAEKPQGEQ...202
204...RVVQFAEKPKGFD...216
188...KIIEFVEKPANPP...200

                N
244...RDKFPGANDFGSEVIPGATS...259
258...KWSYPTSNDFGSEIIPAAID...273
231...DRDENSSHDFGKDLIPKITE...249
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Fig. 3. Alignment of the primary amino acid residues of the large and small subunits of potato ADP-glucose pyrophosphorylase and of *E. coli* ADP-glucose pyrophosphorylase. The order of the alignment is mutated residue identified in this study, potato tuber small subunit, potato tuber large subunit, and *E. coli*. Residues Lys39, Ala44, Tyr114 and Lys195 identified to be important in binding of the allosteric effector fructose, 1,6-bisphophosphate, and substrates ATP and glucose-1-phosphate, respectively, in *E. coli* ADP-glucose pyrophosphorylase are underlined. Residues identified by random mutagenesis of ADP-glucose pyrophosphorylase potato small subunit cDNA which are shown to affect substrate or allosteric binding and the identified substitutions are in bold.

(K_m = 6.4 mM) when compared to wild-type (K_m = 2.1 mM).

The replacement of Ala106Thr causes a more complicated enzymatic interaction by effecting the enzyme's affinity for the substrate, ATP, the cofactor Mg^{2+}, and the allosteric activator 3-PGA. However this substitution had no effect on the enzyme's binding constant for the substrate glucose-1-phosphate. The enzyme has a 8-fold lower affinity to ATP when complexed with the cofactor Mg^{2+} (K_m = 1.38 mM) than the wild-type (K_m = 0.18 mM) and 2.5-fold lower affinity with K^+ ATP (K_m = 0.45 mM) when compared to wild-type (K_m = 0.18 mM). This substituted enzyme also has a 3-fold lower affinity to the cofactor Mg^{2+} (K_m = 6.4 mM) than the wild-type (K_m = 2.1 mM) and a 2.8-fold lower affinity for the allosteric activator 3-PGA ($A_{0.5}$ = 0.48 mM) when compared to the wild-type ($A_{0.5}$ = 0.17 mM).

The fourth mutant class contains an enzyme with altered sensitivity to allosteric effectors. The Pro43Ser substituted enzyme was solely effected in enzyme affinity for the allosteric effectors. Enzyme affinity for the substrates and the co-factor Mg^{2+} were not significantly altered from wild-type (see Table 3). This enzyme has an 8-fold lower affinity for the allosteric activator 3-PGA ($A_{0.5}$ = 1.29 mM) than the wild-type enzyme ($A_{0.5}$ = 0.17 mM). Comparison of various potential allosteric activators demonstrated the mutant enzyme was activated most strongly by 3-PGA with lower levels of activation by fructose 1,6-

Table 3. Kinetic parameters of partially purified wild-type and mutant ADP-glucose pyrophosphorylase: comparison of the wild-type enzyme to the substrate mutant enzymes

	G1P (K_m)	ATP (K_m)	3PGA ($A_{0.5}$)	Mg^{2+} (K_m)	Pi ($I_{0.5}$)*
Wild-type	0.19±0.07	0.18±0.06	0.17±0.02	2.1±0.9	0.17±0.015
Mutant 715 (D252N)	1.21±0.12	0.20±0.05	0.18±0.01	4.5±0.1	0.29±0.02
Mutant 772 (A106T)	0.25±0.04	1.38±0.08	0.48±0.05	6.4±0.1	0.32±0.03†
Mutant 814 (D121N)	1.97±0.05	0.52±0.03	0.28±0.01	6.4±0.8	0.17±0.03
Mutant 947 (P43S)	0.18±0.08	0.29±0.01	1.29±0.02	3.5±0.2	0.81±0.03†

All values given in mM and are the average of at least 2 separate determinations.
* $I_{0.5}$ values determined in 0.125 mM 3-PGA.
† $I_{0.5}$ values determined at the K_m levels for 3-PGA.

diphosphate, phosphoenolpyruvate, and glucose 1,6-diphosphate (data not shown). Sensitivity to Pi inhibition was compared at the $A_{0.5}$ levels exhibited by the mutant and wild-type enzymes. At 1.3 mM 3-PGA the Pro43Ser substituted enzyme showed a $I_{0.5}$ of 0.81 mM Pi while at 0.125 mM 3-PGA the wild-type showed a $I_{0.5}$ of 0.17 mM Pi. Therefore the Pro43Ser substitution reduces the enzyme's affinity to the allosteric activator 8-fold and to the allosteric inhibitor 5-fold.

Glycogen quantitation of null-staining mutants. Level of *in vitro* activity of the enzyme as seen by its ability to produce glycogen was determined by quantifying the level of glycogen in the wild-type and negative staining mutants (Table 2). The wild-type contained glycogen levels of 86.7 mg g^{-1} wet weight, comparable to published levels in other *E. coli* strains of 84.8 mg g^{-1} wet weight (20) while the glycogen deficient strain alone contained glycogen levels below detectable levels (<0.1 mg g^{-1} wet weight). Mutant colonies in Groups I and II had extremely low levels of glycogen compatible with decreased ADP-Glc pyrophosphorylase activities *in vitro* (Table 2).

Assessment of the maximum velocity of the partially purified mutant enzymes under saturation conditions. Decreased affinity for the substrates and allosteric activators may be due to gross structural changes in the mutant enzymes caused by the single amino acid changes. To address this question, the maximum velocity of the mutant enzymes was estimated under saturation conditions using Lineweaver–Burk plots. The overall purity of the partial purified enzyme was estimated by quantitating percent purity from SDS polyacrylamide gels with the computer program Molecule Analyst. Using this approach, the Ala106Thr substituted enzymes showed a substantial reduction in overall enzyme activity with only 49% of the catalytic activity of the wild-type recombinant enzyme. In contrast, enzyme containing the Pro43Ser and Asp252Asn exchange exhibited approximately normal catalytic activity levels whereas the Asp121Asn enzyme showed close to a 2-fold greater level than the recombinant wild-type enzyme.

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