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ADP-Glucose Pyrophosphorylase, a Regulatory Enzyme for Bacterial Glycogen Synthesis

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INTRODUCTION: FUNCTION AND REGULATION OF ADP-Glc PPase IN BACTERIA AND IN PLANTS

Many organisms, including plants, accumulate carbon and energy reserves to cope with starvation conditions temporarily present in the environment (42, 72, 73, 78, 88). The biosynthesis of α-1,4-polyglucans is a main strategy for such metabolic storage. One outstanding advantage in using polysaccharides as reserve compounds is that after their high molecular weights and other physical properties, they have little effect on the internal osmotic pressure in the cell. The metabolic routes for polyglucan accumulation were elucidated after the discovery of nucleoside diphosphate sugars by Luis F. Leloir and coworkers in the 1950s (51). The seminal work of Leloir's group clearly established that biosynthesis and degradation of glycogen occur by different pathways, the former involving the use of an activated form of glucose, specifically UDP-Glc in cells from mammals, fungi, and eukaryotic heterotrophic microorganisms and ADP-Glc in bacteria and photosynthetic eukaryotes (88).

The precise role that the glycogen may play in bacteria is still not clear; however, it was suggested that the accumulation of glycogen by bacteria may give advantages during starvation periods, providing a stored source of energy and carbon surplus (95). In bacteria such as *Bacillus subtilis* and *Streptomyces coelicolor*, glycogen synthesis has been associated with sporulation and the supply of resources necessary to drive differentiation (43, 56, 68), whereas in *Mycobacterium smegmatis*, recycling of the polysaccharide during exponential phase was shown to be essential for growth (7). In *Streptococcus mutans*, it has been shown that a glycogen-like intracellular polysaccharide plays a central role in cariogenesis (92). Also, a relationship between glycogen synthesis, biofilm formation, and virulence has been reported in *Salmonella enteritidis* (9).

The process for the synthesis of storage polysaccharides in bacteria and plants, namely glycogen and starch, respectively, occurs by utilizing ADP-Glc as the glucosyl donor for the elongation of the α-1,4-glucosidic chain (42, 72, 73, 77, 78, 88). Moreover, in these organisms the main regulatory step of the metabolism takes place at the level of ADP-Glc synthesis, a reaction catalyzed by ADP-Glc pyrophosphorylase (ATP:α-D-glucose-1-phosphate adenylyltransferase; EC 2.7.7.27; ADP-Glc PPase): ATP + Glc-1-phosphate ⇔ ADP-Glc + inorganic pyrophosphate. This reaction was first described in soybean (16) and was subsequently found in many bacterial extracts and plant tissues (42, 70, 72, 73, 76–78, 88). The enzymatic reaction takes place in the presence of a divalent metal ion, Mg^{2+}, and it is freely reversible in vitro, with an equilibrium close to 1. The hydrolysis of inorganic pyrophosphate by inorganic pyrophosphatase and the use of the sugar nucleotide for polysaccharide synthesis causes the ADP-Glc synthesis reaction to be essentially irreversible in vivo (42).

Most of the ADP-Glc PPases so far characterized are allosterically regulated by small effector molecules. Although the major activators vary according to the source, they share the characteristic of being intermediates in the major carbon assimilatory pathway in the organism (72, 73, 75, 78, 88). For instance, many of the enzymes from heterotrophic bacteria are activated by metabolites of glycolytic pathways (either the classical Embden-Meyerhof or the Entner-Doudoroff metabolism), such as fructose 6-phosphate, fructose 1,6-bisphosphate, or pyruvate, and inhibited by AMP, ADP, and/or P_i (73, 75, 78). In general, ADP-Glc PPase activators are key metabolites that represent signals of high carbon and energy contents within the cell. The opposite occurs for inhibitors of the enzyme, which are intermediates of low metabolic energy levels.

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These regulatory properties of ADP-Glc PPase, together with the fact that ATP is one of the substrates of the enzyme, have the rationale that synthesis of storage polysaccharides in bacteria and plants will be maximal when cellular carbon and energy are in excess, and vice versa (39, 78, 88).

Cross talk between activators and inhibitors in the ADP-Glc PPase from different sources that renders an amplified response to small changes in concentration of the activator has been described. This response, defined as ultrasensitive behavior (45), is observed because the inhibitor, at higher concentrations, increases the sigmoidicity of the activation curves in spite of decreasing the activity of the enzyme. Detailed studies performed in cyanobacteria have shown that the inhibitor P_i elicits an ultrasensitive response of the enzyme towards 3-phosphoglycerate (3-PGA) activation, which is operative within the cell (27, 28) and allows the enzyme to respond efficiently to minimal changes in 3-PGA levels despite a background of high P_i that may be present inside the cyanobacterial cell (28). Early characterization of the enzyme purified from *Escherichia coli* also showed the interaction between the activator and inhibitors (24).

PHYSIOLOGICAL ROLE

There is strong experimental evidence to support the view that ADP-Glc PPase is a regulatory enzyme on the pathway for bacterial glycogen and plant starch biosynthesis. In *E. coli* and *Salmonella enterica* serovar Typhimurium, several mutants affected in the ability to accumulate glycogen were isolated after chemical mutagenesis, and their ADP-Glc PPases displayed altered regulatory properties (31, 74, 94). It was shown that there was a direct relationship between the affinity of the enzyme for the activator, fructose-1,6-bisphosphate, and the ability of the mutant to accumulate glycogen (79). Similar results were obtained with oxygenic photosynthetic organisms, in which the activator is 3-PGA and the inhibitor is P_i. In the unicellular green alga *Chlamydomonas reinhardtii*, starch-deficient mutants were isolated and shown to have ADP-Glc PPases that could not be activated by 3-PGA (2). Comparable experimental data support the physiological importance of ADP-Glc PPase allosteric regulation in both photosynthetic and nonphotosynthetic tissues from higher plants (26, 53–55, 90, 93). Thus, regulation of ADP-Glc synthesis in bacteria and plants agrees with the generalization that a biosynthetic pathway is effectively regulated at its first unique step.

REGULATORY PROPERTIES AND QUATERNARY STRUCTURE OF ADP-Glc PPases FROM DIFFERENT SOURCES

Based on specificity for activator and inhibitor, ADP-Glc PPases have been grouped into different classes (42, 70, 72, 78, 88). The former classifications can be updated to include nine distinctive classes of ADP-Glc PPases (Table 1) to include recent reports on the properties of the enzymes from gram-positive bacteria (96) and from endosperm tissues of higher plants (30). Also reported in Table 1 is the quaternary structure of the enzymes from different prokaryotic and eukaryotic organisms.

Class I comprises ADP-Glc PPases from bacteria that perform glycolysis (typically enterobacteria: *E. coli*, *S. enterica* serovar Typhimurium), mainly regulated by fructose bisphosphate, the activator, and AMP, the inhibitor (76). The enzyme from class I is encoded by a single gene, giving rise to a native homotetrameric structure (α_4) with a molecular mass of about 200 kDa (Table 1) (42, 73, 75, 88). Other bacteria that perform glycolysis contain ADP-Glc PPases that are allosterically activated by fructose bisphosphate and fructose 6-phosphate and inhibited by AMP and ADP (class II) or exhibit no sensitivity to activator and are inhibited by AMP (class III) (Table 1) (42, 71–73, 88). The enzymes included in class IV are those from bacteria that mainly utilize the Entner-Doudoroff glycolytic pathway, which are distinctively activated by fructose 6-phosphate and pyruvate, with ADP, AMP, and P_i behaving as inhibitors (15, 97). Interestingly, ADP-Glc PPases from organisms using both the Embden-Meyerhoff and the Entner-Doudoroff pathways are activated by the three main effectors: fructose 1,6-bisphosphate, fructose 6-phosphate, and pyruvate (class V, Table 1) (32, 36). As also specified in Table 1, ADP-Glc PPases from *Agrobacterium tumefaciens* (97) and *Rhodobacter sphaeroides* (36) have been characterized as tetramers composed of a single subunit with a molecular mass of about 50 kDa.

Class VI includes ADP-Glc PPases from anaerobic bacteria that are capable of growth under heterotrophic conditions in the dark or being autotrophic in the light and performing anoxygenic photosynthesis (Table 1). These organisms cannot catabolize glucose but grow very well on pyruvate and tricarboxylic acid cycle intermediates as carbon sources and photosynthetic electron donors. Enzymes from class VI are specifically regulated by pyruvate (Table 1) (22, 101).

ADP-Glc PPases grouped as class VII include the enzymes from sporulating bacteria of the genus *Bacillus* (Table 1). These microorganisms accumulate glycogen only during sporulation and in the presence of a carbon source that does not interfere with such a process for survival in hostile environments (96). Under these conditions, the main pathway for carbon utilization is the tricarboxylic acid cycle, which fully metabolizes the by-products of glycolysis (57). It has been determined that in *Bacillus subtilis* and *Bacillus stearothermophilus*, the genes for glycogen synthesis are clustered in one operon, *glgBCDAP* (43, 96). A comparative analysis of the gene clusters showed that *glgC* and *glgD* encode proteins homologous to ADP-Glc PPases from prokaryotes. Thus, the putative GlgC protein from *B. stearothermophilus* has 387 amino acids, with a predicted molecular mass of 43.3 kDa and showing 42 to 70% identity with bacterial ADP-Glc PPases. The GlgD product is a shorter protein (343 amino acids and a predicted molecular mass of 38.9 kDa) with a lower homology to ADP-Glc PPase (20 to 30% identity) (96).

Expression of the *glgC* gene from *B. stearothermophilus* rendered an active recombinant enzyme; whereas GlgD exhibited negligible activity. However, when the *glgC* and *glgD* genes were expressed together, the resulting GlgCD protein exhibited higher affinity for substrates and twofold higher V_{max} in catalyzing ADP-Glc synthesis than GlgC by itself. The different recombinant enzymes from *B. stearothermophilus* were insensitive to regulation by different metabolites typically affecting the activity of other bacterial ADP-Glc PPases (96). Thus, the enzymes grouped in class VII in Table 1 are very distinct from

TABLE 1. Relationships between carbon metabolism and regulatory and structural properties of ADP-Glc PPase from different organisms

Organism	Main carbon utilization	Major reserve polyglucan	ADP-Glc PPase			
			Class	Allosteric regulators[a]		Quaternary structure
				Activator(s)	Inhibitor(s)	
Prokaryotes						
Escherichia coli *Salmonella enterica* serovar Typhimurium *Enterobacter aerogenes*	Embden-Meyerhof pathway (glycolysis)	Glycogen	I	Fru 1,6-bisP	AMP	Homotetramer (α_4)
Aeromonas formicans *Micrococcus luteus* *Mycobacterium smegmatis*	Glycolysis	Glycogen	II	Fru 1,6-bisP, Fru 6-P	AMP, ADP	
Serratia marcescens *Enterobacter hafniae* *Clostridium pasteurianum*	Glycolysis	Glycogen	III	None	AMP	
Agrobacterium tumefaciens *Arthrobacter viscosus* *Chromatium vinosum* *Rhodobacter capsulata* *Rhodomicrobium vannielii*	Entner-Doudoroff pathway	Glycogen	IV	Pyruvate, Fru 6-P	AMP, ADP	Homotetramer (α_4)
Rhodobacter gelatinosa *Rhodobacter globiformis* *Rhodobacter sphaeroides* *Rhodocyclus purpureus*	Glycolysis and Entner-Doudoroff pathways	Glycogen	V	Pyruvate, Fru 6-P, Fru 1,6-bisP	AMP, Pi	Homotetramer (α_4)
Rhodospipillum rubrum *Rhodospirillum tenue*	Tricarboxylic acid cycle Reductive carboxylic acid cycle	Glycogen	VI	Pyruvate	None	
Bacillus subtilis *Bacillus stearothermophillus*	Tricarboxylic acid cycle during sporulation	Glycogen	VII	None	None	Heterotetramer ($\alpha_2\beta_2$)
Cyanobacteria						
Synechococcus sp. strain PCC 6301 *Synechocystis* sp. strain PCC 6803	Oxygen evolving photosynthesis	Glycogen	VIII	3-PGA	Pi	Homotetramer (α_4)
Anabaena sp. strain PCC 7120	Calvin cycle					
Eukaryotes						
Green algae						
Chlorella fusca *Chlorella vulgaris* *Chlamydomonas reinhardtii*	Oxygen evolving photosynthesis Calvin cycle	Starch	VIII	3-PGA	Pi	Heterotetramer ($\alpha_2\beta_2$)
Higher plants						
Photosynthetic tissues						
Leaves of spinach, wheat	Oxygen evolving photosynthesis	Starch	VIII	3-PGA	Pi	Heterotetramer ($\alpha_2\beta_2$)
Arabidopsis, maize, rice	Calvin cycle					
Nonphotosynthetic tissues Potato tubers	Catabolism of sucrose imported from photosynthetic tissues	Starch	VIII	3-PGA	Pi	Heterotetramer ($\alpha_2\beta_2$)
Endosperm of maize, barley and wheat		Starch	IX	None directly, 3-PGA and Fru 6-P reverse inhibitor's effect	Pi, ADP, Fru 1,6-bisP	Heterotetramer ($\alpha_2\beta_2$)

[a] Fru, fructose; P, phosphate; bisP, bisphosphate; Pi, inorganic phosphate.

other ADP-Glc PPases, as they are apparently unregulated enzymes, being the only bacterial ADP-Glc PPases that exhibit a heterotetrameric structure of the type $\alpha_2\beta_2$.

The last group of bacterial ADP-Glc PPases are those from cyanobacteria, prokaryotes that perform an oxygenic photosynthetic process similar to that occurring in plants (class VIII, Table 1). These enzymes have 3-PGA and P_i as the main activator and inhibitor, respectively (13, 40). Remarkably, the specificity for allosteric regulators of the cyanobacterial ADP-Glc PPase is identical to that found in eukaryotic photosynthesizers, such as green algae and higher plants, which are also grouped in class VIII (Table 1) (40, 42). All these photosynthetic organisms utilize the reductive pentose phosphate pathway or Calvin cycle to photoassimilate atmospheric CO_2, rendering 3-PGA as the first intermediate product. P_i under light conditions is utilized to regenerate ATP through photophosphorylation (41). Thus, class VIII ADP-Glc PPases are typically regulated by the 3-PGA/P_i ratio under physiological conditions (41, 42, 73, 78, 88).

Concerning ADP-Glc PPases from nonphotosynthetic tissues of higher plants, two different types can be distinguished (Table 1). The potato tuber enzyme is the best-characterized

ADP-Glc PPase from reserve tissues that are typically activated by 3-PGA and inhibited by P_i and thus grouped as class VIII (Table 1) (5, 37). In addition, the potato enzyme is subject to regulation by a redox mechanism involving Cys-12, with the thioredoxin-mediated reduction of an intermolecular disulfide bridge resulting in activation of the enzyme (3, 20). This was proposed to be operative in different tissues of higher plants (leaves, tuber, fruit, and cotelydons, except in endosperms from monocots). In contrast, the ADP-Glc PPases from bacteria lack a Cys-12 homologous residue (3).

ADP-Glc PPases from reserve tissues of cereals have been reported to exhibit distinctive regulatory properties, mainly related to a lower sensitivity to activators (30, 35, 44, 69, 82, 98). Recently (30), a complete characterization of the ADP-Glc PPase purified from wheat endosperm showed that the enzyme is subject to regulation by the coordinate action of a series of metabolites. The wheat endosperm enzyme is allosterically inhibited by P_i, ADP, and fructose 1,6-bisphosphate. In all cases, inhibition can be reversed by 3-PGA and fructose 6-phosphate, which individually (in the absence of the inhibitors) have no effect on enzyme activity (30). Thus, rather than being an unregulated PPase, this enzyme seems to have distinctive regulatory properties accounting for a class IX group of ADP-Glc PPases (Table 1) that have P_i inhibition as a key signal, as shown in genetically modified plants (90).

Cyanobacterial ADP-Glc PPase occupies a central position with respect to structure/regulation relationships, as its properties are intermediate between those of the bacterial and plant enzymes. Thus, cyanobacterial PPase is homotetrameric in structure, as observed for the protein from other bacteria (Table 1), but it is regulated like and is immunologically more related to the plant enzyme (12, 40). A main difference between the cyanobacterial and plant ADP-Glc PPases is the quaternary structure (38).

SUBUNIT STRUCTURE OF PLANT ENZYMES

Early studies on the spinach leaf ADP-Glc PPase showed the existence of two distinct subunits (62). Other immunological studies in maize endosperm suggested that in both nonphotosynthetic and photosynthetic tissues, the ADP-Glc PPase comprised two subunits that are the products of two genes (77). ADP-Glc PPases from all the eukaryotes characterized so far (starting with the green alga proteins; see Table 1) is composed of α and β subunits to form a heterotetrameric structure (38, 42, 73, 77, 78, 88). In the recombinant potato tuber ADP-Glc PPase, it was shown by N terminus sequencing that the structure is $\alpha_2\beta_2$ (17). For convenience, these subunits were named the small (α subunit, 50 to 54 kDa) and large (β subunit, 51 to 60 kDa) subunits, even though the difference in mass between them in some cases is not more than 1 kDa (63, 64). The small subunit of the higher plant ADP-Glc PPase is highly conserved (85 to 95% identity), whereas the large subunit is less conserved (50 to 60% identity) (91). Nevertheless, both subunits seem to derive from the same ancestor, based on the homology of conserved regions.

IDENTIFICATION OF IMPORTANT AMINO ACID RESIDUES

Chemical modification has been used to identify important amino acids in the ADP-Glc PPases, and site-directed mutagenesis was employed to confirm their roles. Photoaffinity analogs of ATP and ADP-Glc, 8-azido-ATP and 8-azido-ADP-Glc, respectively, were used to identify a residue at the substrate-binding site. When UV light at 257 nm is used to irradiate azido compounds, a nitrene radical is formed, which reacts with electron-rich residues. In the *E. coli* enzyme, it was shown after covalent labeling of these analogs, tryptic digestion, separation, and isolation of the peptides by high-pressure liquid chromatography and subsequent amino acid sequencing that Tyr^{114} was modified (49, 50). Site-directed mutagenesis of this residue showed a marked decrease in affinity for ATP, but it did not seem to be specific only for ATP, since the affinity for Glc 1-phosphate and the activator fructose 1,6-bisphosphate also decreased (49). This residue must be close to the adenine ring of ATP or ADP-Glc but probably also near the Glc 1-phosphate and the fructose 1,6-bisphosphate regulatory sites.

Pyridoxal 5-phosphate (PLP) is a reagent that is able to react with lysine residues to form Schiff bases that can be covalently bonded after reduction with $NaBH_4$. Since PLP may be considered a structural analog of fructose 1,6-bisphosphate and 3-PGA (it activates the ADP-Glc PPases from *E. coli*, *Anabaena* sp., and spinach leaf) (71), it was used to find lysine residues located in those activator sites. In the enzyme from spinach leaf, PLP bound at Lys^{440} very close to the C terminus of the small subunit and also to three other Lys residues in the large subunit. Binding to these sites was prevented by the allosteric effector 3-PGA, which indicated that they are close to or directly involved in the binding of this activator (1, 61). Similar results were obtained with the ADP-Glc PPase from the *Anabaena* sp. In this case, the modified residues were identified as Lys^{419}, which is homologous to Lys^{440} and Lys^{441} in the small subunits of the spinach and potato tuber enzymes, respectively, and Lys^{382} is analogous to Lys^{404} of the potato tuber small subunit. Identification of these residues as regulatory binding sites was confirmed by site-directed mutagenesis of the *Anabaena* ADP-Glc PPase (12, 85).

Mutation of these Lys residues in the potato tuber ADP-Glc PPase revealed that they are also part of the 3-PGA site in heterotetrameric enzymes and that the contribution of these residues to the binding of 3-PGA is additive (4). However, mutation of the small subunits yielded enzymes with less affinity for 3-PGA than homologous mutants of the large subunit. These data indicate that Lys^{404} and Lys^{441} on the potato tuber small subunit are more important than their homologous counterparts on the large subunit, suggesting that the large subunit does not modify the regulatory properties of the small subunit, providing more effective allosteric sites but making the 3-PGA activator sites which are already present in the small subunit more efficient (4).

Chemical modification studies on the *E. coli* enzyme showed that it was covalently modified with $[^3H]$PLP by reduction with $NaBH_4$. It was demonstrated that the PLP could bind to two different lysine residues. Allosteric activators protected binding to Lys^{39}, and substrate ADP-Glc protected binding to Lys^{195} (66, 67). Site-directed mutagenesis of Lys^{39} showed that

this residue is important for the interaction of the activator fructose 1,6-bisphosphate with the enzyme (23). Interestingly, PLP, as an analog of the activator, was reactive with lysine in the N terminus of the *E. coli* enzyme rather than to the C terminus, as in enzymes activated by 3-PGA.

In *E. coli* ADP-Glc PPase, site-directed mutagenesis of Lys^{195} produced enzymes whose K_m for Glc 1-phosphate was 100- to 10,000-fold greater than that of the wild type (34). On the other hand, kinetic constants for ATP, Mg^{2+}, and fructose 1,6-bisphosphate were similar to those of the wild-type enzyme, suggesting that this Lys is specifically involved in the binding of Glc 1-phosphate. Furthermore, the k_{cat} for the glutamine mutant was similar to that of the wild type, ruling out the participation of this residue in the catalytic reaction (34). Site-directed mutagenesis was used to determine the role of this conserved residue in the small (Lys^{198}) and large (Lys^{213}) subunits of the potato tuber ADP-Glc PPase (21). Mutation of Lys^{198} of the small subunit to Arg, Ala, or Glu had little effect on kinetic constants for ATP, Mg^{2+}, activator (3-PGA), and inhibitor (P_i), but the apparent affinity for Glc 1-phosphate decreased 135- to 550-fold. However, similar mutations on Lys^{213} of the large subunit had little effect on the affinity for Glc 1-phosphate. These results indicate that Lys^{198} in the small subunit is directly involved in the binding of Glc 1-phosphate and that the homologous counterpart in the large subunit it is not (21). This is in good agreement with the idea that the large subunit does not have a catalytic role but only a modulatory one (20).

Arginine residues in ADP-Glc PPases were found to be functionally important, as shown by chemical modification with phenylglyoxal (39, 86). Alanine scanning mutagenesis of ADP-glucose pyrophosphorylase from *Anabaena* sp. strain PCC 7120 indicated that Arg^{294} plays a role in inhibition by orthophosphate (86). Recently, it was shown that replacement of this residue with Ala or Gln reversed the pattern of inhibitor specificity; the main inhibitor was NADPH rather than P_i (18). All of these results suggest that the positive charge of Arg^{294} may not be specifically involved in orthophosphate binding but that it plays a role in determining inhibitor selectivity.

Alanine scanning mutagenesis of the arginine residues located in the N terminus of the enzyme from *Agrobacterium tumefaciens* demonstrated the presence of separate subsites for the activators fructose 6-phosphate and pyruvate (29). The R32A mutant enzyme had reduced affinity for fructose 6-phosphate (11.5-fold) and behavior identical to the wild-type enzyme with respect to pyruvate activation. Both the R33A and R45A mutant enzymes had higher activity than the wild-type enzyme in the absence of activators and no response to fructose 6-phosphate, but partial activation by pyruvate and desensitization to phosphate inhibition (29).

Random mutagenesis experiments were performed on the potato tuber ADP-Glc PPase to find residues that are important for the enzyme. Even though several residues were found, some of them did not show a very big decrease in activity or a very specific effect. The most interesting finding was that Asp^{403} (in the article it is described as Asp^{413}) in the small subunit is important for activation by 3-PGA (33). This residue is adjacent to the lysine that is responsible for PLP binding and 3-PGA activation. Mutation of residue Asp^{253} on the small subunit showed a specific effect on the apparent affinity for Glc 1-phosphate, but the K_m only increased 10-fold (48). Interestingly, this residue is conserved in the sugar nucleotide pyrophosphorylases that have been crystallized and whose structure has been solved when an alignment is made according the secondary-structure elements (19). This residue seems to be close the substrate site without a direct interaction with Glc 1-phosphate.

PREDICTION OF THE STRUCTURE OF ADP-Glc PPases

Information about the three-dimensional structure of any ADP-Glc PPase would be tremendously helpful for structure-function relationship studies. Unfortunately, it is not currently available. For that reason, several methods to predict the structure have been applied (19, 81). A modified hydrophobic cluster analysis (52) was applied to several ADP-Glc PPases from different sources representing different classes according to homology of subunits and tissue, i.e., *E. coli*, *Anabaena*, *Chlamydomonas*, potato (*Solanum tuberosum* L.) tuber small subunit and different large subunits from maize embryo, maize shrunken 2, and *Arabidopsis thaliana*. Hydrophobic cluster analysis showed that the ADP-Glc PPases were extremely similar in the distribution and pattern of the clusters, even between bacterial and plant enzymes. This strongly suggests that the ADP-Glc PPases have a common folding pattern despite a different quaternary structure ($\alpha_2\beta_2$ in plants and α_4 in bacteria) and specificity for the activator.

If the ADP-Glc PPases from different sources have a similar three-dimensional structures, their secondary-structure predictions should be similar. All the sequences mentioned above, and also those from *A. tumefaciens*, *Bacillus stearothermophilus*, and *Rhodobacter sphaeroides*, were analyzed with the PHD program to predict the secondary structure (81). The alignment helped to establish a structure for regions where the predictions were not conclusive for one of the enzymes but very clear for the rest (19). A similar alignment of representative bacterial ADP-Glc PPases from each class is shown in Fig. 1. The small-subunit sequences of the enzymes from *C. reinhardtii* and potato tuber were also included for comparison with the proteins from uni- and pluricellular eukaryotes, respectively (Fig. 1). From these analyses, a general structure that fits all of these proteins was postulated (Fig. 2). There are also biochemical data that support the model (19).

Controlled proteolysis experiments were in good agreement with the model. The exposed loops would be more sensitive to proteolytic cleavage, and the studies confirmed that the proteases analyzed cut in sites predicted to be loops (19). The only exception is the α-helix predicted near the C terminus on the *Anabaena* enzyme (Fig. 2). Since this is an insertion (20 amino acids) that is absent in the *E. coli* enzyme and is not predicted to be buried by the PHD program, it is most likely that this helix is not part of the core but part of a loop in a domain of eight β-sheets (Fig. 2).

Loops are prone to have insertions and deletions in homologous proteins that do not alter the structure. In our model, all the insertions and deletions observed fell in loops (Fig. 2). The conserved amino acids known to have specific roles in the binding of substrates (*E. coli* Tyr^{114} and Lys^{195}) and activators (*E. coli* Lys^{39} and *Anabaena* Lys^{382} and Lys^{419}) are located in

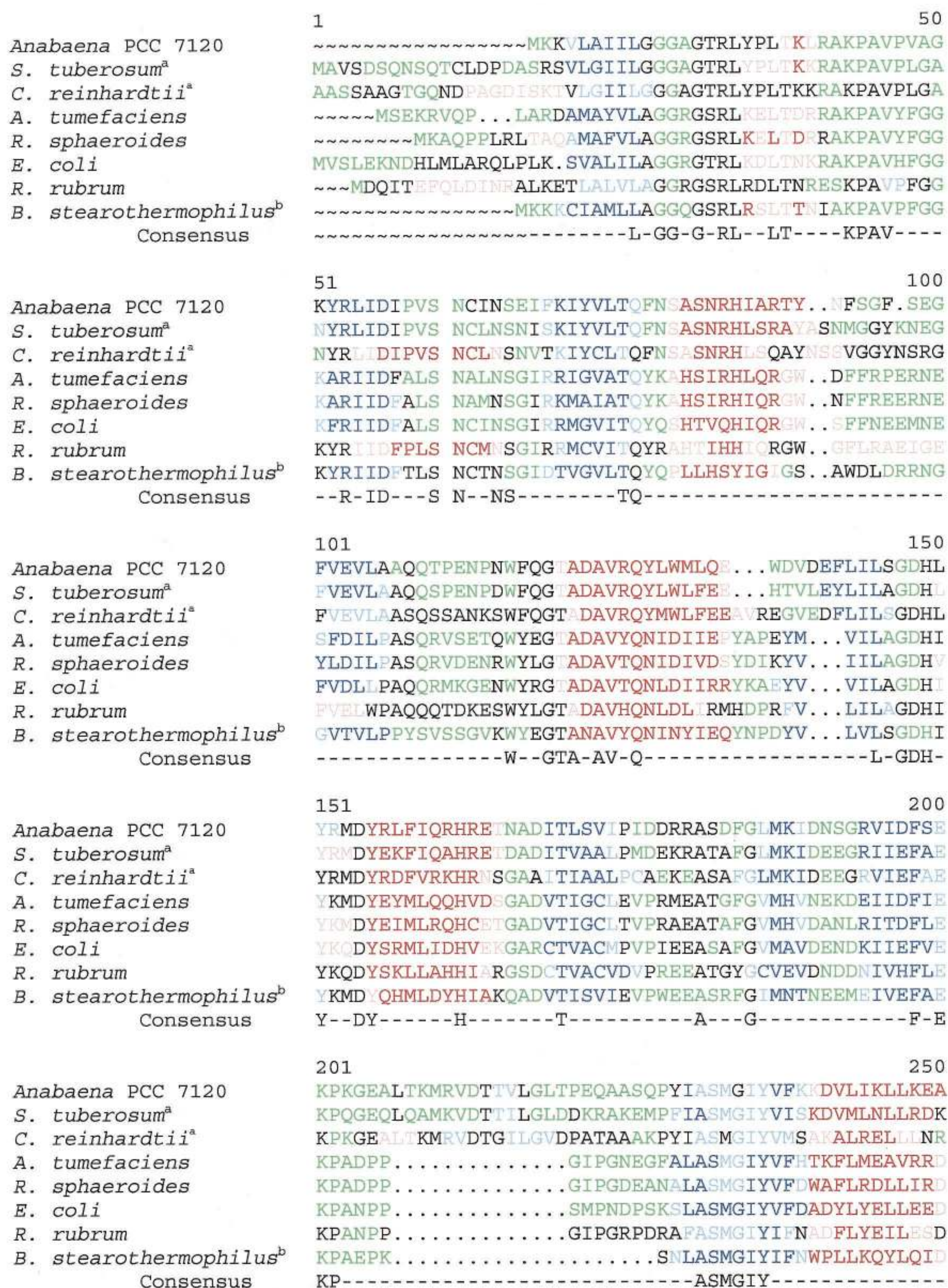


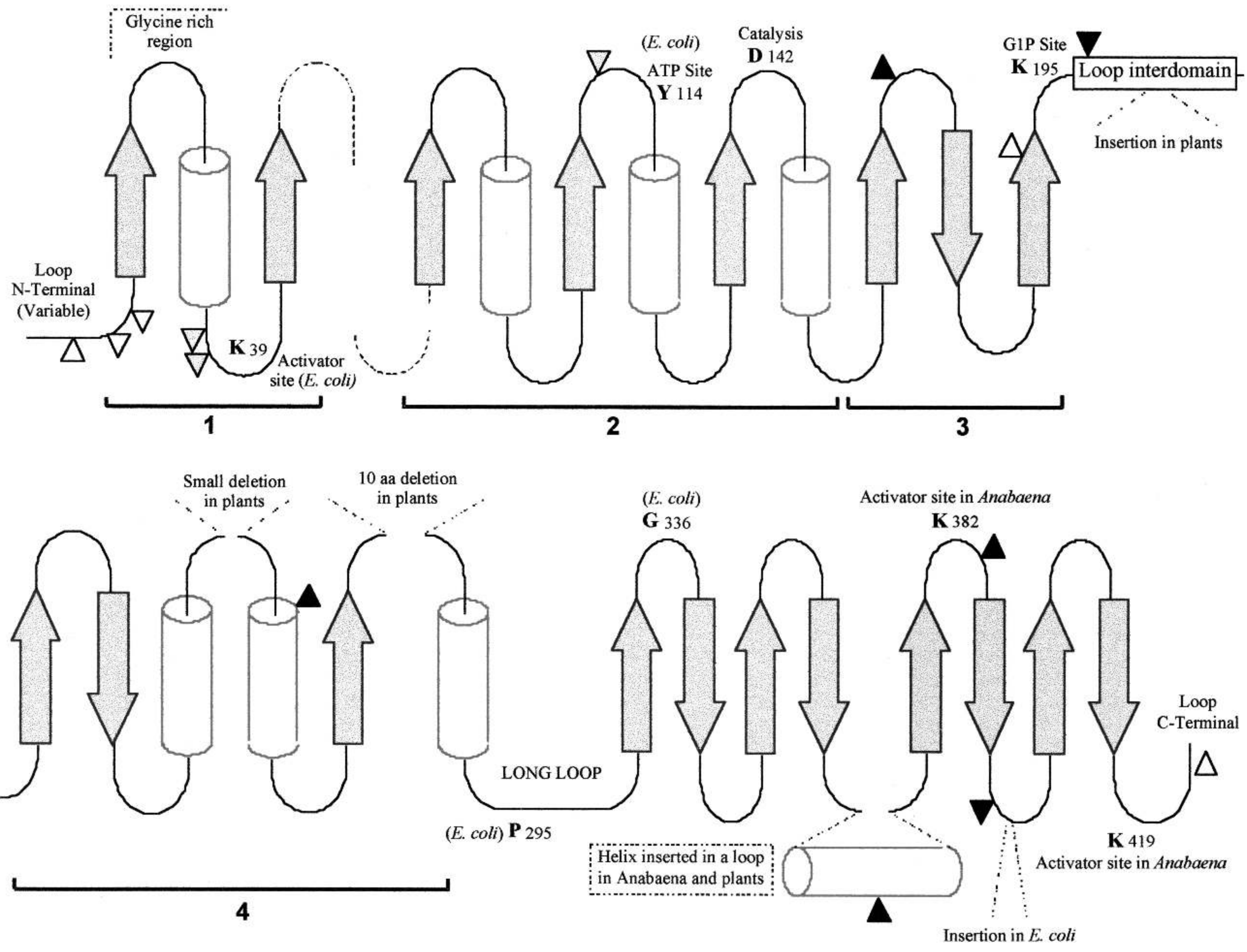
FIG. 1. Alignment of ADP-Glc PPases from different classes. Amino acid alignment was performed with the program PILEUP from the Wisconsin package (http://www.gcg.com). The alignment was fine tuned manually based on the secondary structure of each enzyme as predicted by the PHD program (81). Residues in blue and red were predicted to be β-sheets and α-helixes, respectively; pale shades indicate a lower level of confidence. Green residues were predicted to be neither of these (loops). In black are residues for which the PHD program could not make a prediction. Insertions and deletions were introduced to maximize the alignment of both primary and secondary structure. a, sequence of the small (catalytic) subunit; b, sequence of the subunit encoded by *glgC* (catalytic).

```
                           251                                              300
Anabaena PCC 7120          LE.RT...DFGKEIIPDAAK.DHNVQAYLF...........DDYWEDIGT
S. tuberosum^a             FPGAN...DFGSEVIPGATSLGMRVQAYLY...........DGYWEDIGT
C. reinhardtii^a           MPGAN...DFGNEVIPGAKDAGFKVQAFAF...........DGYWEDIGT
A. tumefaciens             AADPTSSRDFGKDIIPYIV.EHGKAVAHRFADSCVRSDFEHEPYWRDVGT
R. sphaeroides             AEDPNSSHDFGHDLIPAIV.KNGKAMAHRFSDSCVMTGLETEPYWRDVGT
E. coli                    DRDENSSHDFGKDLIPKIT.EAGLAYAHPFPLSCVQSDPDAEPYWRDVGT
R. rubrum                  ALNEASQHDFGRDIIPSQV.GKARIVAHRFSQSCVYSVGRREPYWRDVGT
B. stearothermophilus^b    NANPHSSHDFGKDVIPMLLREKKRPFAYPF...........EGYWKDVGT
         Consensus         --------DFG---IP----------A----------------YW-D-GT

                           301                                              350
Anabaena PCC 7120          IEAFYNANLALTQQPMPPFSFYDEEAPIYTRARYLPPTKLLDCH......
S. tuberosum^a             IEAFYNANLGITKKPVPDFSFYDRSAPIYTQPRYLPPSKMLDAD......
C. reinhardtii^a           VEAFYNANLALTDPEKAQFSFYDKDAPIYTMSRFLPPSKVMDCD......
A. tumefaciens             IDAYWQANIDLTDV.VPDLDIYDKSWPIWTYAEITPPAKFVHDDEDRRGS
R. sphaeroides             IDAFWQANIDLTDF.TPKLDLYDREWPIWTYSQIVPPAKFIHDSENRRGT
E. coli                    LEAYWKANLDLASV.VPELDMYDRNWPIRTYNESLPPAKFVQDRSGSHGM
R. rubrum                  VDAYWSANIDLVSV.TPALDLYDADWPIWTYQMQRPPAKFVFDTDERRGM
B. stearothermophilus^b    VKSLWEANMDLLDE.NNELDLFDRSWRIYSVNPNQPP.QYISPEAE....
         Consensus         ------AN-------------D----I-------PP------------

                           351                                              400
Anabaena PCC 7120          VTESIIGEGCILK.NCRIQHSVLGVRSRIETGCMIEESLLMGADFYQASV
S. tuberosum^a             VTDSVIGEGCVIK.NCKIHHSVVGLRSCISEGAIIEDSLLMGADYYETDA
C. reinhardtii^a           VNMSIIGDGCVIKAGSKIHNSIIGIRSLIGSDCIIDSAMMMGSDYYETLE
A. tumefaciens             AVSSVVSGDCII.SGAALNRSLLFTGVRANSYSRLENAVVLPS.......
R. sphaeroides             AISSLVSGDCIV.SGSEIRSSLLFTGCRTHSYSSMSHVVALPH.......
E. coli                    TLNSLVSGGCVI.SGSVVVQSVLFSRVRVNSFCNIDSAVLLPE.......
R. rubrum                  AKDSLVSAGCIV.SGGAVTGSLLFNDVRVNSYSSVIDTVILPM.......
B. stearothermophilus^b    VSDSLVNEGCVV.EGT.VERSVLFQGVRIGKGAVVKESVIMPG.......
         Consensus         ---S-----C----------S---------------------------

                           401                                              450
Anabaena PCC 7120          ERQCSIDKGDIPVGIGPDTIIRRAII..DKNARIGHDVKIINKDNVQEAD
S. tuberosum^a             DRKLLAAKGSVPIGIGKNCHIKRAII..DKNARIGDNVKIINKDNVQEAA
C. reinhardtii^a           E..CEYVPGCLPMGVGDGSIIRRAIV..DKNARIGPKCQIINKDGVKEAN
A. tumefaciens             ............VKIGRHAQLSNVVI..DHGVVIPEGLIVGEDPELDAKR
R. sphaeroides             ............VTVNRKADLTNCVL..DRGVVVPEGLVIGQDAEEDARW
E. coli                    ............VWVGRSCRLRRCVI..DRACVIPEGMVIGENAEEDARR
R. rubrum                  ............GDIGRHARLTKCIL..DTGCRIPEGLVIGEDPILDAKR
B. stearothermophilus^b    ............AAVSEGAYVERAIVTPDS.IIPPHSSVCPEDADD....
         Consensus         --------------------------D--------------------

                           451
Anabaena PCC 7120          RESQGFYIRS..GIVVVLKNAVITDGTII~~~~~~
S. tuberosum^a             RETDGYFIKS..GIVTVIKDALIPSGIII~~~~~~
C. reinhardtii^a           REDQGFVIKD..GIVVVIKDSHIPAGTII
A. tumefaciens             .....FR.RTESGICLITQSMIDKLDL~~~~~~~~
R. sphaeroides             .....FR.RSEGGIVLVTQDMLDARARALN~~~~~
E. coli                    .....FY.RSEEGIVLVTREMLRKLGHKQER~~~~
R. rubrum                  .....FH.VTEQGITLVTPDRLALL
B. stearothermophilus^b    .............VVLVTAEWLKQSNEETARKDEA
         Consensus         -----------------------------~~~~~~
```

FIG. 1—*Continued.*

loops. The residues Pro[295] and Gly[336], which seem to be located in a region important for the regulation of the *E. coli* enzyme, are also in loops (25, 59). The amino acid Asp[142] in the *E. coli* enzyme was identified as a catalytic residue (19), and it is also present in a loop.

A structure usually observed in proteins that bind nucleotides is also predicted in this model. Region 1 has a Gly-rich loop after a β-sheet, which is similar to a P loop in protein kinases or nucleotide binding sites (84), and region 2 has three β-sheets and helices that are compatible with the Rossman fold (80).



Glycine rich
region
Loop
N-Terminal
(Variable)
K 39
Activator
site (E. coli)
1
(E. coli)
ATP Site
Y 114
Catalysis
D 142
2
G1P Site
K 195
Loop interdomain
Insertion in plants
3
Small deletion
in plants
10 aa deletion
in plants
4
LONG LOOP
(E. coli) P 295
(E. coli)
G 336
Helix inserted in a loop
in Anabaena and plants
Activator site in Anabaena
K 382
Loop
C-Terminal
K 419
Activator site in Anabaena
Insertion in E. coli

Thus, regions 1 and 2 comprise a putative domain or subdomain that binds ATP. Moreover, Tyr^{114}, which was shown to be reactive to the azido analog of ATP (49, 50), is in this region.

α/β structures generally have a very particular topology regarding the loops. Some of them are "functional" because they carry residues important for binding and catalysis, and others are just "connectors" because they only connect one helix with the next sheet. It has been observed that functional loops are the ones that are located at the C-terminal end of the β-sheets (10). Supporting the model, those loops in regions 1, 2, and 3 are the ones that bear the most conserved amino acids. Hence, this is compatible with the idea that the ATP would be facing the "top" of the structure depicted in Fig. 2. Moreover, amino acid residues located at the loops that are at the N terminus of the β-sheets in regions 2 and 3 are not conserved at all. The exception is in region 1; however, there is evidence by chemical modification and site-directed mutagenesis that this loop interacts with the activator fructose 1,6-bisphosphate in the *E. coli* ADP-Glc PPase (23).

The first pyrophosphorylase domain to be crystallized and solved was present in a bifunctional enzyme that is the product of the gene *glmU* (11). One domain of the GlmU protein is a UDP-*N*-acetylglucosamine pyrophosphorylase, and the other is an acetyltransferase. Later, other pyrophosphorylase domain structures were solved (8, 46, 65, 89). All these structures verify the predicted secondary-structure model of the ADP-Glc PPase (19). Regions 2, 3, and 4 are virtually identical. In region 4, the only difference is that two β-sheets were predicted rather than one because of the presence of a Gly (breaker). In the *N*-acetylglucosamine uridyltransferase, only one sheet is bent because of a Gly. Region 1 is very similar; there is a P-loop-like structure, but our model predicted an extra β-sheet. It is possible that the prediction is wrong or that different sugar nucleotide pyrophosphorylases vary in this region. When we predicted the secondary structure of GDP-mannose PPases, TDP-Glc PPases, CDP-Glc PPases, and UDP-Glc PPases, this was the region with the greatest variability. For this reason, the topology of the loop where Lys^{39} is present cannot be ascertained. To support the idea that the sugar-nucleotide pyrophophorylases have a similar catalytic domain, it was demonstrated that the homologous Glc 1-phosphate site is present in the GDP-mannose PPase from *Pseudomonas aeruginosa* (58).

The homology between ADP-Glc PPases and the *N*-acetylglucosamine uridyltransferase is extremely low. However, an alignment could be done using the predicted structure to match helices and sheets. Then, several residues were found conserved, all in loops that face the substrate in the *N*-acetylglucosamine uridyltransferase (Fig. 2). Lys^{195}, the Glc 1-phosphate binding site in ADP-Glc PPases, is present in the *N*-acetylglucosamine uridyltransferase but shifted one position.

CATALYTIC RESIDUES

Despite the identification of several important residues on the structure of the ADP-Glc PPases, only recently was an amino acid identified as being mainly involved in catalysis (19). Comparison with the three-dimensional structures of known pyrophosphorylase domains and prediction of the structure led to the discovery of highly conserved residues throughout the superfamily of pyrophosphorylases despite the low homology.

FIG. 2. Prediction of secondary structure of ADP-glucose pyrophosphorylases. The secondary structure of various ADP-Glc PPases from bacteria as well as plants was predicted with the PHD program (81). The secondary structures align very well (19) with the sequences of UDP-*N*-acetylglucosamine pyrophosphorylase (11)and TDP-glucose pyrophosphorylase (8). Sequences shown as arrows are predicted to be β-pleated sheets, and sequences shown as cylinders are predicted to be α-helices. These structures are interconnected with amino acid sequences indicated as being neither α-helices or β-pleated sheets and are possibly random structures or loops. They are shown as lines. White triangles indicate areas where proteinase K hydrolyzes the *E. coli* enzyme (99). Black triangles indicate where the *Anabaena* ADP-Glc PPase is partially proteolyzed by trypsin, and gray triangles indicate partial hydrolysis of the *E. coli* enzyme by trypsin (unpublished data). The proteolysis results suggest that the areas sensitive to proteases are exposed random structures (loops). Residues K^{39}, Y^{114}, and K^{195} are the amino acids in the *E. coli* ADP-Glc PPase that bind the activator fructose 1,6-bisphosphate and the substrates ATP and Glc 1-phosphate, respectively. D^{142} is the amino acid shown to be a catalytic residue in the *E. coli* enzyme. P^{295} and G^{336} are amino acids that, when mutated, affect the allosteric properties of the ADP-Glc PPase (59, 60). Regions 1, 2, and 3 form the putative catalytic domain, and region 4 may also be part of the catalytic domain, as suggested by alignment with the crystal structures of UDP-*N*-acetylglucosamine pyrophosphorylase (11) and TDP-glucose pyrophosphorylase (8).

Asp[142] in the *E. coli* ADP-Glc PPase was predicted to be close to the substrate site. Site-directed mutagenesis of this residue to Ala and Asn confirmed that the main role of Asp[142] is catalytic (19). Kinetic analysis showed a decrease in specific activity of four orders of magnitude, whereas other kinetic parameters showed no significant changes.

In the pyrophosphorylase domain of the GlmU enzyme, it was proposed that Arg[18] could be a catalytic residue (11). Even though this residue seems to be important in ADP-Glc PPases, it is not clear if it is directly involved in the catalytic reaction. Mutagenesis of the homologous Arg[25] in the enzyme from *Agrobacterium tumefaciens* yielded an enzyme with an activity reduced by two orders of magnitude (29). Generally, it is expected that more dramatic effects would occur after mutation of catalytic residues.

DOMAIN CHARACTERIZATION

The central region of the protein has been identified as a substrate binding and catalytic domain by secondary-structure prediction, alignment with other sugar nucleotide pyrophosphorylase enzymes, and further site-directed mutagenesis. It has been proposed that the N and C termini are responsible for the distinctive regulatory properties of the different classes of ADP-Glc PPases (6). This is evident for the ADP-Glc PPases from oxygenic photosynthetic organisms because key residues for the regulation have been found on the C terminus of plant and cyanobacterial ADP-Glc PPases (4, 14, 18, 33, 85, 86). Also, several modifications on the C terminus caused modifications in the regulation of plant enzymes (26, 83). Residues that are critical for the binding of the activators have been found only on the N terminus of enzymes from heterotrophic bacteria (23). Two allosteric mutants (P295S and G336D) were found and characterized in the C terminus of the *E. coli* enzyme, but they had higher rather than decreased apparent affinities for the activator, which indicates that they are probably not involved in the binding of the regulators (59, 60). However, those mutants indicate the importance of the C terminus in regulation.

Recent experiments with chimeric enzymes between the ADP-Glc PPase from *E. coli* and *A. tumefaciens* suggest that the C terminus of the *A. tumefaciens* enzyme determines the high apparent affinity for the activator pyruvate, but the residues critical for the fructose 6-phosphate selectivity do not lie in this region (6). This agrees with site-directed mutagenesis experiments that suggested that the sites for the two activators are separate or overlap only partially (29). Experiments with the chimeric enzymes also supported the idea that the C terminus of the *E. coli* enzyme largely contributes to determining the selectivity for the activator fructose 1,6-bisphosphate. Since it has been found previously that Lys[39] in the *E. coli* enzyme interacts with the allosteric activator (23), it is very possible that the regulation is determined by a combined arrangement between the N and C termini.

The N-terminal region of the ADP-Glc PPase, which is predicted to be a loop, may play a role as an "allosteric switch" to regulate enzyme activity. This loop possibly interferes with the transition between two different conformations of the enzyme (activated and nonactivated). A shorter N terminus may favor a conformation of the enzyme that facilitates activation. This was observed when the enzyme from *E. coli* and the small subunit from the potato tuber enzyme were truncated by 11 amino acids (5, 99, 100). It was necessary to remove at least 11 amino acids from the *E. coli* enzyme to observe this effect. When a truncation of 10 amino acids was generated in the small subunit of the potato tuber enzyme, the apparent affinity for the activator 3-PGA increased and the apparent affinity for the inhibitor P_i decreased (5). Similar results were observed when the large (modulatory) subunit was truncated by 17 amino acids in the N terminus (47).

EVOLUTION OF ADP-Glc PPases

ADP-Glc PPases seem to have a common pyrophosphorylase domain with other sugar nucleotide pyrophosphorylases, as suggested previously. ADP-Glc PPases are generally bigger because they have an extended C terminus (120 to 150 amino acids) and a slightly longer N terminus (10 to 40 amino acids). Differences in selectivity for the regulators of the ADP-Glc PPases play a key metabolic role in the organisms that use ADP-Glc for synthesis of polysaccharides as carbon and energy storage. It is possible that a common enzyme ancestor evolved to other forms having different regulatory properties accommodating different metabolic environments and developed into several classes of ADP-Glc PPase (Table 1).

It is very possible that a fragment of ≈150 amino acids at the C terminus was acquired to make it a regulated enzyme and/or to improve the rudimentary regulation that was already present. The other sugar nucleotide pyrophosphorylases are not considered allosteric enzymes, and this extended C terminus is either lacking or part of a completely different domain to form a bifunctional enzyme (11, 87). It is not known whether the regulatory sites are located in the same or distinct domains in the protein structure, but it seems that the C terminus plays an important role in all the ADP-Glc PPase classes. Some of these enzymes are relatively nonspecific in selectivity for allosteric regulators, which indicates a certain flexibility to undergo evolutionary changes for adaptation to a certain metabolism. This is evident because minimal changes in the protein sequence can alter the specificity for the regulators. A single mutation (K419Q) on the *Anabaena* ADP-Glc PPase changed the preference of the activator from 3-PGA to fructose 1,6-bisphosphate (14).

Experiments with directed molecular evolution demonstrated that a few mutations of the small subunit from the potato tuber enzyme could alter the selectivity for activators of the homotetrameric form (α_4) (83). Construction of chimeric enzymes showed that a single "crossover" between two genes rendered two ADP-Glc PPases that would belong to different classes than their parents. From enzymes of class I (*E. coli*) and class IV (*A. tumefaciens*), two ADP-Glc PPases that could be included as class V (chimeric enzyme AE) and class VI (chimeric enzyme EA) were found (6). It has been observed that this plasticity is also present in the inhibitor site of the *Anabaena* enzyme. Mutants R294A, R294E, and R294Q changed the selectivity from P_i to NADPH (18). Unfortunately, the structure-function relationships of the regulatory site(s) in heterotrophic bacteria are far from clear. A more comprehensive characterization of the structure of the allosteric sites will be very important to understand the evolutionary mechanism.

Another important step in the evolution is the appearance of the β (large) subunit in eukaryotes, most probably by gene duplication. This allowed further divergence and specialization to obtain different polypeptides, a catalytic and a modulatory subunit. The catalytic subunit had more constraints to its evolution, and that could be why they show very high homology even among different plants. Moreover, cyanobacterial homotetrameric enzymes show higher homology with the small subunits from plants than the small and large subunits show between themselves. The small subunit kept the catalytic function but lost the ability to be activated efficiently in the absence of the large subunit. Replacement of several amino acids showed that this process could be reversed in vitro (83). The large subunits might have evolved to satisfy different requirements in the tissues (88). Later, the catalytic subunit on certain tissues might have acquired the ability to be regulated by thioredoxin (3).

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