Aspartate Residue 142 Is Important for Catalysis by ADP-glucose Pyrophosphorylase from Escherichia coli

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Received for publication, August 2, 2001, and in revised from, September 20, 2001
Published, JBC Papers in Press, September 20, 2001, DOI 10.1074/jbc.M107408200

Structural prediction of several bacterial and plant ADP-glucose pyrophosphorylases, as well as of other sugar-nucleotide pyrophosphorylases, was used for comparison with the three-dimensional structures of two crystallized pyrophosphorylases (Brown, K., Pompeo, F., Dixon, S., Mengin-Lecreulx, D., Cambillau, C., and Bourne, Y. (1999) EMBO J. 18, 4096–4107; Blankenfeldt, W., Asuncion, M., Lam, J. S., and Naismith, J. H. (2000) EMBO J. 19, 6652–6663). This comparison led to the discovery of highly conserved residues throughout the superfamilly of pyrophosphorylases despite the low overall homology. One of those residues, Asp142 in the ADP-glucose pyrophosphorylase from Escherichia coli, was predicted to be near the substrate site. To elucidate the function that Asp142 might play in the E. coli ADP-glucose pyrophosphorylase, aspartate was replaced by alanine, asparagine, or glutamate using site-directed mutagenesis. Kinetic analysis in the direction of synthesis of pyrophosphorylases of the purified mutants showed a decrease in specific activity of up to 4 orders of magnitude. Comparison of other kinetic parameters, i.e. the apparent affinities for substrates and allosteric effectors, showed no significant changes, excluding this residue from the specific role of ligand binding. Only the D142E mutant exhibited altered $K_m$ values but none as pronounced as the decrease in specific activity. These results show that residue Asp142 is important in the catalysis of the ADP-glucose pyrophosphorylase from E. coli.

Glycogen and starch, which consist of mainly α1,4-polyglucans, encompass most of the energy reserves in organisms. The biosynthesis of these polysaccharides proceeds from the formation of the glucosyl donor, ADP-glucose (ADP-Glc). ADP-glucose pyrophosphorylase (ADP-Glc PPase, EC 2.7.7.27) catalyzes the synthesis of ADP-Glc and pyrophosphate (PPi) from ATP and glucose 1-phosphate (Glcp-1-P) (1). This reversible reaction is the key regulatory step in the production of glycogen in bacteria and starch in plants (2–5).

ADP-Glc PPase has been isolated and characterized from various sources. Most of the enzymes are allosterically regulated by the glycolytic intermediates of the major pathway of carbon assimilation in the organism (6). The regulatory effectors include the activator fructose 1,6-bisphosphate (FBP) and the inhibitor AMP for an enzyme from enteric bacteria, e.g. Escherichia coli (7), and the activator 3-phosphoglycerate and the inhibitor inorganic phosphate for enzymes from plants and other photosynthetic organisms, e.g. algae and cyanobacteria (4, 5). Active ADP-Glc PPase forms a tetramer of about 200 kDa, which is either homomeric (α$_4$) in bacteria (8, 9) or heteromeric (α$_1$β$_2$) in plants (10, 11). The emergence of two different subunits in plants, designated as small and large based on disparities in molecular weight, likely arose from the need for tissue-specific regulation (5, 12). Also, other studies show that each subunit has a separate function: the small subunit is catalytic while the large subunit is a mediator of the allosteric regulation of the small subunit (13).

The three-dimensional structure of an ADP-Glc PPase has not yet been resolved by x-ray crystallography; however, many structural studies have been performed using other approaches. A combination of techniques, such as chemical modification and site-directed mutagenesis, has been applied to study the ADP-Glc PPase enzyme from E. coli. Azido-based photoaffinity analogs of the ATP substrate and the ADP-Glc substrate (pyrophosphorylase direction) were used to probe the substrate-binding site. Analysis of the covalently labeled enzyme revealed Tyr$^{114}$ as the modified residue, and subsequent site-directed mutagenesis of the residue showed a marked decrease in affinity for ATP, as well as a decrease in affinity for Glc-1-P and FBP (14). The residue likely interacts with the adenine ring of ATP but is not crucial for the binding of the substrate. Pyridoxal-5-phosphate, a mild reagent that modifies lysines within ligand-binding sites upon reduction by NaBH$_4$, is an analog of the activator from E. coli, FBP. Reaction of this modifying agent with the enzyme labels two different lysines, Lys$^{195}$ and Lys$^{395}$ that are blocked by the inclusion of FBP and of the substrate ADP-Glc, respectively (15, 16). Mutation of Lys$^{395}$ showed that this residue is important in the interaction of the activator FBP with the enzyme (17). Mutagenesis of the Lys$^{195}$ residue produced enzymes with drastically increased $K_m$ values for Glc-1-P, while the other kinetic constants and the $k_{cat}$ were not altered, showing clearly that this residue is involved in the binding of Glc-1-P in both bacterial and plant enzymes (18, 19).

These techniques have also been used to probe the regulatory sites of ADP-Glc PPases from photosynthetic organisms. Studies of pyridoxal-5-phosphate-modified residues in the ADP-Glc PPases from spinach leaves and the cyanobacterium Anabaena PCC 7120 have implicated specific lysine residues located in the C terminus that are involved in the binding of the activator 3-phosphoglycerate (20–22). These residues are not present in the bacterial enzymes. Site-directed mutagenesis of these homologous residues in ADP-Glc PPase from Anabaena and from the heterotetrameric enzyme from potato (Solanum tuberosum...
L.) tuber specifically affects the apparent affinity for activator (22, 23). The results from the above structural studies show that functionally important residues located in the active site are conserved in ADP-Glc PPases from different organisms; however, the residues related to the regulation of these enzymes seem to have diverged.

Protein sequence alignments of ADP-Glc PPases show a high degree of conservation (85–95% similarity) among enzymes from bacteria and among the small subunits of plants; however, the similarity between the two groups is about half (30–40%) (24). Inclusion of the large subunits from plants in the alignment with the small subunits improves the percent similarity slightly (50–60%) from the comparison between bacteria and the small subunits. The degree of similarity can be increased for enzymes from bacteria and the small and large subunits when limiting the comparison to the region containing roughly the first 200 residues (Fig. 1). Within this region, many residues are highly conserved among the various ADP-Glc PPases. Moreover, the region contains at least a few key residues conserved throughout other enzymes that catalyze the synthesis of a nucleoside diphosphate sugar from a sugar-phosphate and a nucleoside triphosphate (19). Although these enzymes have been characterized thoroughly by the above methods, a residue involved in the catalytic reaction has not been isolated. The three-dimensional structure of an ADP-Glc PPase will likely be of help in this endeavor, and work on the crystallography is in progress. In the meantime, a prediction of the structure of ADP-Glc PPase is needed to interpret the relationship between the available genetic and biochemical data and the structure of the enzyme, and to possibly identify putative residues involved in catalysis.

In the current study, we present the secondary structure predictions and alignments of several ADP-Glucose PPases by the methods of PHD and a modified “hydrophobic cluster analysis.” These predictions were compared with the solved three-dimensional structures of other enzymes, dTDP-glucose pyrophosphorylase (RmlA (25)) and N-acetylglucosamine 1-phosphate uridylytransferase (GluM (26)), that catalyze the synthesis of a nucleoside diphosphate sugar. Although sequence homology of these enzymes is low in comparison to the ADP-Glc PPases, we found a definite conservation of secondary structure. Asp142 in E. coli is absolutely conserved throughout these sugar-nucleotide pyrophosphorylases and is located in a predicted loop. This same loop in RmlA and GlmU is located in a region containing the 10% sucrose.

Expression and Purification of Wild-type and Mutant Enzymes

The wild-type and D142E plasmids were expressed in E. coli bacterial strain BL21(DE3). A single colony of transformed cells was grown in Luria broth (1 liter) at 37 °C up to an A600 of 0.6. Induction was initiated by the addition of isopropyl-β-D-thiogalactoside (1 mM final concentration), with subsequent incubation at 25 °C for 4 h. The cells were then harvested and resuspended in storage buffer containing 20 mM potassium phosphate (pH 7.5), 5 mM dithiothreitol, and 1 mM EDTA (about 5 mg of cell/1 ml of buffer). All subsequent protein purification steps were conducted at 0–4 °C. The suspensions were sonicated for three 30-s intervals with cooling on ice between sonications. The sonicated suspensions were centrifuged for 15 min at 15,000 × g, and the supernatants were retained. An ammonium sulfate cut (30–60%) of the supernatants was performed, with centrifugation of the cut samples for 20 min at 20,000 × g. The 30–60% ammonium sulfate pellets were resuspended in 3 ml of storage buffer and desalted on Bio-Rad 10 DG chromatography columns equilibrated with buffer A (50 mM HEPES (pH 7.5), 10% sucrose, 2.5 mM dithiothreitol, and 1 mM EDTA). The desalted samples were applied individually to a Mono Q HR 10/10 column equilibrated with buffer A. The column was washed with 2 column volumes of buffer A, and the samples were eluted with a linear KC1 gradient (160 ml, 0–0.5 M) in buffer A. Fractions of 4 ml were collected and those containing activity were desalted in buffer A minus the 10% sucrose.

The D142N and D142A mutant plasmids were expressed in E. coli bacterial strain AC70R1-504, which is deficient in endogenous ADP-Glc PPase activity. Transformed cells (1 liter) were induced as described for the plasmid pMA10 (13). The mutant enzymes were purified as above.

The specific activity in the crude extracts of the wild type, D142N, D142E, and D142A enzymes were 45, 0.009, 0.5, and 0.01 units/mg, respectively. The enzymes remained stable during the purification, and the total yield was 24, 36, 37, and 21%. Since the proteins were overexpressed, to reach homogeneity (a single band stained with Coomassie Blue after 2 μg were run in an SDS-PAGE) it was necessary to purify them only 4.6-3.5-5.4, and 4-fold, respectively.

Enzyme Assay

Assay A: Pyrophosphorylation—Formation of [32P]ATP from [32P]PPP, in the direction of pyrophosphorylation at 37 °C was determined by the method of Shen and Preiss (29). The reaction mixtures contained 80 mM HEPES (pH 7.0), 7 mM MgCl₂, 1.5 mM [32P]PPP (1500–2500 cpm/nmol), 2 mM ADP-Glc, 1 mM FBP, 4 mM NaF, and 0.05 mg/ml bovine serum albumin, plus enzyme in a total volume of 250 μl. The reaction mixtures for the D142E mutant were similar, except they contained 10 mM MgCl₂, 2 mM [32P]PPP, and 3 mM FBP to compensate for increased K₉ values as overt.

Assay B: Synthesis—Formation of [14C]ADP-glucose from [14C]Glc-1-P in the synthesis direction at 37 °C was determined by the method of Preiss et al. (7). The reaction mixtures consisted of 100 mM HEPES (pH 7.6), 6 mM MgCl₂, 0.5 mM [14C]Glc-1-P (1000 cpm/nmol), 1 mM ATP, 2 mM FBP, 0.0015 units/μl pyrophosphatase, and 0.2 mg/ml bovine serum albumin, plus enzyme in a total volume of 100 μl. The reaction mixtures for the D142E mutant were similar, except they contained 12 mM MgCl₂, 3 mM [14C]Glc-1-P (300 cpm/nmol), 6 mM ATP, and 5 mM FBP. When measuring the kinetic value of the inhibitor, AMP, for the wild-type and D142A and D142N mutants, 1.5 mM FBP was present in the assay mixture. The FBP concentration in the same inhibitor assay mixture was increased to 3 mM for the D142E mutant. One unit of enzyme activity in the above assays is equal to 1 μmol of product, either [32P]ATP or [14C]ADP-glucose, formed per minute at 37 °C.
Kinetic Characterization

The kinetic data were plotted as initial velocity (nmol/min) versus substrate or effector concentration (mM). The kinetic constants were acquired by fitting the data with a nonlinear least square formula and the Hill equation using the program Origin® 5.0. The Hill coefficient, \( n_H \), and the kinetic constants, \( K_{	ext{S,0.5}} \), \( A_{	ext{S,0.5}} \), and \( I_{	ext{S,0.5,5}} \), which correspond to the concentration of substrate, activator, or inhibitor giving 50% of the maximal velocity, activation, or inhibition, respectively, were calculated from the Hill plots.

Protein Assay

Protein concentration during enzyme purification was measured by using bicinchoninic acid reagent (30) from Pierce Chemical Co., with bovine serum albumin as the standard. Protein concentration of the purified enzymes was determined by UV absorbance at 280 nm using an extinction coefficient of 1.0.

Protein Electrophoresis and Immunoblotting

Protein purification was monitored by SDS-PAGE as described by Laemmli (31), utilizing 4–15% Tris-HCl pre-cast gradient polyacrylamide gels from Bio-Rad. Perfect™ protein markers were used for molecular weight standards. Following electrophoresis, protein bands were either visualized by staining with Coomassie Brilliant Blue R-250 or electroblotted onto a Protran™ (Schleicher & Schuell) nitrocellulose membrane. The nitrocellulose membrane was subsequently treated with affinity purified anti-\( E. coli \) B strain AC70R1 ADP-Glc PPase IgG (9). The resulting antigen-antibody complex was visualized by treatment with alkaline phosphatase-linked goat anti-rabbit IgG, then staining with BM purple AP-substrate precipitating reagent from Roche Molecular Biochemicals.

Stability of the Enzymes to Heat

An aliquot, 15 μg of the purified enzymes was diluted (1:2) in a 0.5-ml microcentrifuge tube containing the following enzyme dilution buffer: 50 mM HEPES (pH 8.0), 10% sucrose, 5 mM MgCl₂, 0.1 mM EDTA, and 2 mg/ml bovine serum albumin (total volume: 30 μl). The heat treatment was performed at 55 °C for 5 min with subsequent cooling on ice. Aliquots were then removed to measure the activity using the Hill plots.

Structure Prediction Analysis

The amino acid sequence of the ADP-Glc PPases was analyzed by a profile neural network program (PHD program (32)) to predict the secondary structure and the probability for the residues to be exposed. Sequences were aligned manually based on the primary and predicted secondary structure. A modified two-step method of hydrophobic cluster analysis was used to enhance the alignment (33). The original method plotted clusters of amino acids as an α-helix, and the hydrophobic ones were highlighted and encircled to signal the presence of a hydrophobic cluster. In our method, we encircled clusters of residues predicted as “buried” by the PHD program. To expedite this process, we developed a program in Pascal language. This modified method of hydrophobic cluster analysis proved to be more accurate in finding similar clusters in these homologous proteins.

RESULTS

Prediction of the Structure and Alignment of ADP-Glc PPases—The catalytic reactions of RmlA and GlmU are similar to ADP-Glc PPase. The three-dimensional structures of the former enzymes have been solved (25, 26), but the homology to the ADP-Glc PPases is low: 15% identity between the \( E. coli \) ADP-Glc PPase and the pyrophosphorylase domain of GlmU. To verify that ADP-Glc PPases share a common structure and to improve the protein sequence alignment, the secondary structures of several ADP-Glc PPases were predicted. For this prediction, ADP-Glc PPases were selected from different sources, i.e. \( E. coli \), Anabaena, Chlamydomonas reinhardtii, potato tuber “small” subunit, and different “large” subunits from maize embryo, maize shrunken 2, and Arabidopsis thaliana. Each protein represented various classes according to the homology of the subunits and the types of regulation and tissue. The first step was to confirm that the ADP-Glc PPases from different sources share a common structure despite their diversity. A modified method of hydrophobic cluster analysis (33) was applied to these enzymes. Hydrophobic cluster analysis showed that the ADP-Glc PPases are extremely similar in the distribution and pattern of the hydrophobic clusters even between bacterial and plant enzymes (data not shown). This strongly suggests that the ADP-Glc PPases share a common folding pattern, despite differences in quaternary structure (\( \alpha_\beta \) in plants and \( \alpha_\gamma \) in bacteria) and in specificity for the activator.

If the ADP-Glc PPases from different sources have a similar three-dimensional structure, their secondary structure predictions should also be similar. All the sequences mentioned above, including those from Agrobacterium tumefaciens, Bacillus stearothermophilus, and Rhodobacter sphaeroides, were analyzed using the PHD program to predict the secondary structure (32). Processing several sequences helped to establish a structure for aligned regions where the confidence of the prediction for α-helices or β-sheets was low for one of the enzymes but high for the rest (Fig. 1). Also of importance was the prediction of loops, which are assigned by the PHD program when the likelihood of either α-helices or β-sheets is low. To enhance the prediction of the loops, we used the following biochemical and genetic data.

(i) Exposed loops are more sensitive to proteolytic cleavage. Proteolytic studies on ADP-Glc PPase confirmed that proteases cut in sites predicted as loops or very close to them (Fig. 1). The exceptions were two cuts in an α-helix predicted near the C-terminal of the Anabaena enzyme (data not shown) and in a β-sheet (Met181-Ala282, Fig. 1). Since the α-helix is an insertion (20 amino acids) that is absent in the enzyme from \( E. coli \) and is not predicted as buried by the PHD program, it is most likely that this helix is not part of the core but rather a part of a loop in a domain of 8 β-sheets. The β-sheet, which is the second one in region 3, is probably in an exposed region. To support this hypothesis, in GlmU the equivalents of the second and third β-sheets of region 3 are not part of the core of the protein (26).

(ii) During evolution, loops in homologous proteins are prone to insertions and deletions without altering the overall structure of the proteins. In the prediction, all of the insertions and deletions observed among sequences from different sources fell in the predicted loops (Fig. 1).

(iii) The conserved amino acids known to have specific roles in the binding of substrates (\( E. coli \) Tyr114 and Lys195) and of activators (\( E. coli \) Lys39, Anabaena Lys382 and Lys419) are located in the predicted loops. Residues Pro295 and Gly336, which are located in a region that is apparently important for the regulation of the \( E. coli \) enzyme (34, 35), are also in loops.

(iv) It has been observed in catalytic αβ/δ domains that functional loops are located at the C terminus of the β-sheets. The loops after these C termini in regions 2, and 3 contain the most amino acids that are conserved among the ADP-Glc PPases. Moreover, amino acid residues located at loops that are at the N terminus of β-sheets in regions 2 and 3 are not conserved at all. The only exception is in region 1. However, there is evidence from chemical modification and site-directed mutagenesis that this loop interacts with the activator FBP in the ADP-Glc PPase from \( E. coli \) (16, 17).

Taking the above information into consideration, the protein sequences of the ADP-Glc PPases were aligned manually with the sequences of RmlA and of the pyrophosphorylase domain of GlmU. The secondary structure of the predicted model is extremely similar to the secondary structures of these structurally solved proteins.
Expression and Purification of the Wild-type and Mutant Enzymes—The expression of the Asp142 mutant enzymes was similar to the wild-type enzyme as determined by Western blot (data not shown). Thus, either the BL21(DE3) or the AC70R1-504 expression systems were efficient at overexpressing the recombinant ADP-Glc PPases. The drawback to using the BL21(DE3) expression system is the presence of an endogenous ADP-Glc PPase activity, which might interfere with an accurate determination of the kinetic parameters of the mutants D142N and D142A because of the decrease in their catalytic activity. For this reason, mutants D142N and D142A were analyzed from purified samples that were expressed in AC70R1-504 cells, which are deficient in endogenous ADP-Glc PPase activity, as described under “Experimental Procedures.” The homogeneous enzymes were then used for kinetic analyses.

Kinetic Characterization of the Wild-type and Mutant Enzymes—The mutant enzymes were assayed both in the synthesis

Expression and Purification of the Wild-type and Mutant Enzymes—The expression of the Asp142 mutant enzymes was similar to the wild-type enzyme as determined by Western blot (data not shown). Thus, either the BL21(DE3) or the AC70R1-504 expression systems were efficient at overexpressing the recombinant ADP-Glc PPases. The drawback to using the BL21(DE3) expression system is the presence of an endogenous ADP-Glc PPase activity, which might interfere with an accurate determination of the kinetic parameters of the mutants D142N and D142A because of the decrease in their catalytic activity. For this reason, mutants D142N and D142A were analyzed from purified samples that were expressed in AC70R1-504 cells, which are deficient in endogenous ADP-Glc PPase activity, as described under “Experimental Procedures.” The background activity measured from crude extracts of AC70R1-504 cells transformed with a control plasmid that has no insert (pMAB5) was negligible and comparable to the background obtained when no extract was added to the reaction (data not shown). Despite the low activity of the D142N and D142A mutants, they were still at least 36- and 19-fold higher, respectively, than the background in the same assay of the crude extracts (data not shown).
sis and the pyrophosphorolysis directions, as described under “Experimental Procedures” (assays A and B). To compare the kinetic parameters of the mutant enzymes with the wild-type, the values of the kinetic parameters reported here for the wild-type are in good agreement with those previously reported (e.g. Ref. 18).

In the direction of synthesis, the apparent substrate affinities \( S_{0.5} \) for the D142N and D142A mutants were not significantly different from the values measured for the wild-type (Table I). In contrast, the substrate values for the D142E mutant were altered in comparison to the wild-type, showing a 47-fold increase for Glc-1-P \( S_{0.5} \), an 11.5-fold increase for ATP \( S_{0.5} \), and a 2-fold increase for Mg\(^{2+} \) \( S_{0.5} \) (Table I). The D142N mutant also had an increased Hill coefficient \( (n_H) \) for Glc-1-P (2-fold), showing increased cooperativity for this substrate.

The mutation of Asp142 to Asn and Ala did not seem to affect the ability of the mutant enzymes to bind the activator, FBP. The FBP \( A_{0.5} \) values for the D142E mutant and ATP \( A_{0.5} \) values for the D142A mutant were increased 6- and 7-fold, respectively, whereas the wild-type showed a decrease of around 2 orders of magnitude when compared to the D142A, D142E, and D142N mutants retained 98, 96, and 52% of the initial activity, respectively. The homology with ADP-Glc PPase have been crystallized, and their structures have been solved (RmlA and GlmU). However, the homology with ADP-Glc PPase is very low and computer modeling is not possible. To study the structure-function relationship of a putative catalytic domain of the ADP-Glc PPase from E. coli, we made a prediction of the secondary structure, and the helices, loops, and sheets were matched with the structures of RmlA and GlmU. The prediction was extremely similar to these proteins, enhancing the significance of highly conserved residues that were isolated. These residues were found to be in key regions of particular motifs (Fig. 1). Interestingly, structures usually observed in proteins that bind nucleotides were found to be compatible with the prediction. Region 1 (Fig. 1) has a glycine-rich loop after a \( \beta \)-sheet that is similar to the "P loop" in protein kinases or nucleotide-binding sites (36), and region 2 has three predicted \( \beta \)-sheets and helices that are compatible with the Rossman fold (37). 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 (38, 39), is in this region. These predictions are in excellent agreement with the three-dimensional structures of the RmlA and GlmU enzymes.

The secondary structure of the predicted model is extremely similar to the secondary structure of RmlA and of the pyrophosphorylase domain of the bifunctional enzyme from GlmU. Regions 2, 3, and 4 are virtually identical. In region 4, the only difference is that two \( \beta \)-sheets were predicted rather than one because of the presence of Gly\(^{214} \) (breaker, Fig. 1). In GlmU, this is only one \( \beta \)-sheet, which is bent because of a glycine and is part of the substrate site where the sugar-phosphate rests. Possibly, this motif is typical of the pyrophosphorolase family.

There are other loops in the structures of GlmU and RmlA that face their respective substrates. In the prediction of the
Role of Asp\textsuperscript{142} in ADP-glucose Pyrophosphorylase Catalysis

### TABLE II

| Kinetic parameters of E. coli wild-type and mutant ADP-Glc PPases in the pyrophosphorolysis direction |
|---------------------------------------------------------------|
| **Reactions** performed at 37 °C using assay A as described under "Experimental Procedures." The concentration of Mg\textsuperscript{2+} listed in the table is the total reaction mixture concentration. The data represent the mean of two independent experiments with the difference expressed as a ± deviation. **V**\textsubscript{max} was determined in the presence of saturated conditions of substrates and activator (FBP). The assay conditions to determine the **V**\textsubscript{max} of the D142E mutant were altered to compensate for the increased **S**\textsubscript{0.5} and **A**\textsubscript{0.5} values as described under "Experimental Procedures." Duplicates of the **V**\textsubscript{max} determinations differed less than 5% and were in good agreement with the **V**\textsubscript{max} obtained in the saturated region of the substrate curves. |

| **ADP-Glc** | **PPi** | **Mg\textsuperscript{2+}** | **FBP** | **V**\textsubscript{max} |
|-------------|---------|-----------------|--------|-----------------|
| **S**\textsubscript{0.5} | **n**\textsubscript{H} | **S**\textsubscript{0.5} | **n**\textsubscript{H} | **A**\textsubscript{0.5} | **n**\textsubscript{H} | **Units/mg** | **Fold decrease** |
| wt | 0.11 ± 0.01 | 1.8 | 0.11 ± 0.03 | 0.8 | 1.61 ± 0.05 | 2.5 | 0.015 ± 0.001 | 1.8 | 210 |
| D142E | 0.46 ± 0.02 | 2.3 | 1.2 ± 0.2 | 1.3 | 3.9 ± 0.2 | 2.0 | 0.20 ± 0.04 | 2.2 | 2.14 | 100 |
| D142A | 0.054 ± 0.002 | 1.9 | 0.05 ± 0.01 | 1.1 | 1.8 ± 0.1 | 2.6 | 0.081 ± 0.004 | 1.9 | 0.031 | 6,500 |
| D142N | 0.18 ± 0.02 | 1.8 | 0.18 ± 0.08 | 1.6 | 1.7 ± 0.1 | 2.7 | 0.088 ± 0.002 | 1.8 | 0.020 | 10,500 |

structure of the ADP-Glc PPases we found that there are highly conserved residues in these homologous loops (Fig. 1). Some of them have already been assigned a function. For instance Lys\textsuperscript{195}, the Glc-1-P-binding site in ADP-Glc PPases, is present in the third loop of region 3 and interacts with the sugars-phosphate in both RmlA and GlmU. The homologous lysine is present in RmlA and in GlmU but shifted one position in the latter. The fourth loop in region 2 (Fig. 1) has residues that are conserved in the ADP-Glc PPases and that interact with the dTTP or UTP in RmlA and GlmU, respectively. It contains Tyr\textsuperscript{114}, which has been proposed to be close to the ATP-binding site. Also, the last residue of this loop is Gly\textsuperscript{186} which is part of the motif “GTAD” that is highly conserved in ADP-Glc PPases. Most probably the function of this loop is to confer the specificity for the nucleotide since these residues interact with the base. Asp\textsuperscript{142} in ADP-Glc PPase from E. coli is another one of these highly conserved residues that is located in a loop predicted to be near the substrates by comparison with RmlA and GlmU. In this study the function of this residue was analyzed by site-directed mutagenesis. Replacement of Asp\textsuperscript{142} resulted in a significant decrease in catalysis in either direction of product formation. This effect on catalysis was reduced by conserving the negative charge (Tables I and II), indicating that the charge of this residue plays an important role in the catalytic reaction. This is the first time that a residue involved in the catalysis of an ADP-Glc PPase enzyme has been characterized. Although the activity of the D142N and D142A mutants was reduced by 4 orders of magnitude, we were still able to obtain data on the other kinetic parameters of these mutant enzymes. Measuring the effect of mutations on catalytic residues can be limiting when the activity of the mutant enzyme(s) falls below the sensitivity of the assay. Blankenfeldt et al. (25) mutated the corresponding Asp residue in RmlA based on their determination of the three-dimensional structure of the enzyme and their results show a decrease in catalytic activity of at least 3 orders of magnitude for the mutated enzymes. However, they were not able to measure any other kinetic parameters, providing them with only partial information about this residue in RmlA. In this study, the kinetic parameters for the substrates and allosteric effectors were not altered very much for the D142A and D142N mutants, indicating that the main function of Asp\textsuperscript{142} is not related to their binding.

Even though mutation of Asp\textsuperscript{142} most significantly affected the catalysis of ADP-Glc PPase from E. coli, to a lesser extent the mutation affected the regulation of the D142E and D142N mutants (Table I). As discussed above, the **A**\textsubscript{0.5} for FBP of D142E increased 17-fold and the **I**\textsubscript{50} for AMP of D142N increased 25-fold over wild-type. These changes can be attributed to a suboptimal interaction between the regulatory and the catalytic sites through a disruption of the local environment surrounding Asp\textsuperscript{142}. The mutation of Asp\textsuperscript{142} to Glu does not alter the charge. However, there is an extra methylene in Glu which probably interferes with the normal interaction of this negatively charged residue with those residues that might be important for regulation. In fact, the activator of ADP-Glc PPase from E. coli interacts with Lys\textsuperscript{195}, which was predicted to be in a loop by the PHD program (Fig. 1). This loop is predicted to be near Asp\textsuperscript{142} in the tertiary conformation of the enzyme from the alignment with the proteins RmlA and GlmU. Since the regulation of the ADP-Glc PPase enzyme from E. coli is allosteric, any changes in the affinity for one effector can alter the affinity for the other. Changes in the affinity for the activator FBP could also lead to the observed changes in the inhibition by AMP. This allosteric relationship has been studied in E. coli (40) and in other ADP-Glc PPase enzymes as well (e.g.Refs. 4, 41, and 42), demonstrating the sensitivity of each effector to changes in the concentration of the other.

The exact role Asp\textsuperscript{142} plays in the catalytic mechanism of ADP-Glc PPase from E. coli is unknown. A detailed chemical mechanism of the residues involved in the catalysis of ADP-Glc PPase has not been elucidated; however, the kinetic reaction has been studied. A kinetic mechanism for ADP-Glc PPase was first analyzed in Rhodospirillum rubrum, from which an Ordered Bi Bi mechanism is favored over a Theorell-Chance mechanism (43). The kinetic mechanism of ADP-Glc PPase from E. coli B was found to be similar to R. rubrum, reinforcing a sequential binding of ATP followed by glucose 1-P (44). Study of other sugar-nucleotide PPase mechanisms has also indicated a sequential binding order of substrates (e.g.Refs. 45–47). The solution of the three-dimensional structures of the RmlA and GlmU enzymes, which share a similar reaction to ADP-Glc PPases of forming a sugar-nucleotide product, provides some insight to the results of this study. Based on their results from the crystallized RmlA, Blankenfeldt et al. (25) proposed a chemical mechanism that proceeds by an S\textsubscript{N2} reaction, in which the Asp\textsuperscript{142} residue is directly involved (Asp\textsuperscript{142} in RmlA). They suggest that the Asp activates the α-phosphate of the nucleotide for nucleophilic attack by the sugar-phosphate. In contrast, the pyrophosphorylase domain from the solved GlmU structure shows the homologous Asp to be located in a position that is closer to the ribose moiety of the nucleotide, and the authors suggest that it is involved in the binding of the UTP substrate (26). This contradicts the findings for RmlA and does not support our results for the site-directed mutagenesis of Asp\textsuperscript{142}. Furthermore, our results show this residue to be important in both directions of the catalytic reaction, strongly suggesting the involvement of Asp\textsuperscript{142} in the catalysis of the enzyme.

The three-dimensional structure of RmlA supports our results showing for the first time a residue involved in the catalysis of an ADP-Glc PPase enzyme. The secondary structure of RmlA, as well as of the GlmU enzyme, is very similar to the
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predictions for the ADP-Glc PPases. In particular, the alignment of these enzymes shows a pattern of conserved residues among the different pyrophosphorylases (Fig. 1). This suggests that there is a sharing of a common pyrophosphorylase domain among these enzymes. Within this domain, there are also residues particular to each type of pyrophosphorylase (e.g., the Tyr\textsuperscript{114} residue from E. coli which interacts with the adenine-ring of the ATP substrate for ADP-Glc PPase (14)). Interestingly, most of the residues involved in the regulation of the ADP-Glc PPases are located outside this conserved domain. As was mentioned earlier, this area has diverged even among the class of ADP-Glc PPases. It might be suggested that the variation in the sizes of the N- and C-terminal regions among these different classes of enzyme is related to the specific regulation, if any, for individual classes. Further studies of the ADP-Glc PPases and the other sugar-nucleotide pyrophosphorylases are needed to fully comprehend their relationships with one another.

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