Purification and Characterization of Phosphomannose Isomerase-Guanosine Diphospho-d-mannose Pyrophosphorylase

A BIFUNCTIONAL ENZYME IN THE ALGINATE BIOSYNTHETIC PATHWAY OF PSEUDOMONAS AERUGINOSA*

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We report here the purification and characterization of phosphomannose isomerase-guanosine 5'-diphospho-d-mannose pyrophosphorylase, a bifunctional enzyme (PMI-GMP) which catalyzes both the phosphomannose isomerase (PMI) and guanosine 5'-diphospho-d-mannose pyrophosphorylase (GMP) reactions of the Pseudomonas aeruginosa alginate biosynthetic pathway. The PMI and GMP activities co-eluted in the same protein peak through successive fractionation on hydrophobic interaction, ion exchange, and gel filtration chromatography. The purified enzyme migrated as a 56,000 molecular weight protein on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the native protein migrated as a monomer of 54,000 molecular weight upon gel filtration chromatography. The apparent $K_m$ for d-mannose 6-phosphate was 3.03 mM, and the $V_{max}$ was 830 nmol/min/mg of enzyme. For the GMP forward reaction, apparent $K_m$ values of 20.5 μM and 29.5 μM for d-mannose 1-phosphate and GTP, respectively, were obtained from double reciprocal plots. The GMP forward reaction $V_{max}$ (5,680 nmol/min/mg of enzyme) was comparable to the reverse reaction $V_{max}$ (5,170 nmol/min/mg of enzyme), and the apparent $K_m$ for GDP-d-mannose was determined to be 14.2 μM. Both reactions required Mg$^{2+}$ activation, but the PMI reaction rate was 4-fold higher with Co$^{2+}$ as the activator. PMI (but not GMP) activity was sensitive to dithiothreitol, indicating the involvement of disulfide bonds to form a protein structure capable of PMI activity. DNA sequencing of a cloned mutant algA gene from P. aeruginosa revealed that a point mutation at nucleotide 961 greatly decreased the levels of both PMI and GMP in a crude extract.

The leading cause of mortality in patients afflicted with cystic fibrosis is chronic pulmonary infection by Pseudomonas aeruginosa (1). The pathogenicity of P. aeruginosa in the cystic fibrosis lung is greatly enhanced by the production of alginate, a linear co-polymer of β-1,4-linked D-mannuronic acid and variable amounts of its C-5 epimer L-guluronic acid (2). This exopolysaccharide has been shown to inhibit the phagocytosis of P. aeruginosa (3-7) and to interfere with the action of antibiotics (8, 9). Strains of Pseudomonas which produce alginate (Alg⁺) form mucoid colonies but revert to the nonmucoid form upon continued propagation in the laboratory (10, 11). Our laboratory is engaged in a study of the biochemistry and genetics of the alginate pathway in P. aeruginosa with the goal of isolating and characterizing each biosynthetic enzyme involved in alginate synthesis. These studies could lead to the development of nontoxic inhibitors of the alginate biosynthetic enzymes which can eliminate the alginate barrier, thereby making P. aeruginosa more susceptible to antibiotic therapy in the cystic fibrosis lung.

The pathway for alginate synthesis in P. aeruginosa was originally described by Piggot et al. (12). These authors reported that PMI³, GMP, and GDP-M dehydrogenase enzymatic activities (Fig. 1) were present at low levels in alginate-producing strains of P. aeruginosa. Padgett and Phibbs (13) showed that the second enzymatic step, phosphomannomutase, was low but detectable in mucoid strains of P. aeruginosa, and that this activity was consistently higher in mucoid strains than in nonmucoid strains. Our laboratory has demonstrated that each of these enzymatic steps of the proposed alginate pathway (Fig. 1) is present in extremely low amounts in mucoid alginate-producing P. aeruginosa (14). The enzyme catalyzing the fourth step of alginate synthesis, GDP-mannose dehydrogenase (EC 1.1.1.132), has been purified and characterized in our laboratory by Roychoudhury et al. (15).

Darzins et al. (16) isolated the AlgA gene from a P. aeruginosa genomic library and determined that this gene complemented Escherichia coli and P. aeruginosa PMI mutant strains to mucoidy. Gill et al. (17) showed that IPTG induction of

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1 The abbreviations used are: PMI, phosphomannose isomerase; GMP, GDP-d-mannose pyrophosphorylase; FDP, d-fructose 6-phosphate; MiP, d-mannose 1-phosphate; GDP-M, GDP-d-mannose; IPTG, isopropyl β-d-thiogalactopyranoside; HPLC, high performance liquid chromatography; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PAGb, polyacrylamide gel electrophoresis; MOPS, 4-morpholinopropanesulfonic acid.
algA and SDS-PAGE of the cell extracts resulted in the appearance of a 56,000 molecular weight polypeptide and a 20-fold increase in PMI activity. Further studies in our laboratory by Sá-Correia et al. (14) demonstrated that both PMI and GMP activities were greatly elevated in crude extracts of *P. aeruginosa* containing the overexpressed AlgA protein. They went on to show that both activities co-eluted during fractionation of cell extracts containing overexpressed AlgA protein.

In this study, we report that the algA gene codes for an alginase biosynthetic enzyme which harbors two enzymatic activities: PMI and GMP. The enzyme has been purified and the kinetic parameters for its inherent PMI and GMP activities have been determined. Further evidence for the bifunctional nature of this unique enzyme has been obtained through the sequencing of a mutant algA gene and the study of the altered PMI-GMP enzyme that it encodes.

### EXPERIMENTAL PROCEDURES

#### Materials

SDS, acrylamide, bisacrylamide, Coomassie Blue G-250, protein buffer, pH 7.0, 1 mM CoCl₂, 1 mM NAD⁺, 10 mM MgCl₂, and 4 units of phosphoglucose isomerase and glucose-6-phosphate dehydrogenase in a total volume of 1 ml. The rate of NAD⁺ reduction was monitored at 340 nm at 25 °C in a Gilford model 2600 spectrophotometer.

GMP activity was assayed in the reverse direction by the method of Munch-Petersen (21) using a modified activity mixture containing 100 mM MOPS buffer, pH 7.0, 10 mM MgCl₂, 1 mM NAD⁺, 1 mM ADP, 4 mM d-glucose, 1 mM GDP-M, 1 mM PP₆, and 5 units each of nucleoside-5'-diphosphate kinase, hexokinase, and glucose-6-phosphate dehydrogenase. The rate of NAD⁺ reduction was monitored at 340 nm at 25 °C.

GMP activity was assayed in the forward direction by coupling the reaction to GDP-M dehydrogenase (Fig. 1). GDP-M dehydrogenase was purified from *P. aeruginosa* as described by Roychoudhury et al. (15). The assay mixture contained 100 mM Tris-HCl, pH 8.0, 1 mM MIP, 1 mM GTP, 10 mM MgCl₂, 2 mM NAD⁺, and 500 μg (180 millimolar) of GDP-M dehydrogenase. The rate of NAD⁺ reduction was monitored at 340 nm at 25 °C.

**Purification of PMI-GMP**

**Step 1: Preparation of Cell Extracts** — *P. aeruginosa* 8822 containing the recombinant plasmid pAD4038, which has the algA gene under control of the tac promoter, was grown in 2 × 1 liters of L broth containing 450 μg/ml carbenicillin and 1 mM IPTG at 37 °C with vigorous shaking (250 rpm). After an 8-h growth period, the cells were harvested by centrifugation at 8,000 × g for 10 min, washed once with 0.9% NaCl, and resuspended in 5 ml of lysis buffer (100 mM MOPS, pH 7.0, 10 mM MgCl₂, 15% glycerol, 1 mM phenylmethylsulfonyl fluoride, 2 mM DTT). The cells were sonicated in an oscillator (Heat Systems-Ultrasonic, Inc.) and centrifuged at 40,000 × g for 20 min. The resulting supernatant was then centrifuged at 100,000 × g for 1 h, and the clarified supernatant was used for PMI-GMP purification in Step 2.

**Step 2**—The supernatant from Step 1 was made 500 mM with respect to K₂HPO₄ and 5 ml was injected into a 150 × 2.15-mm Bio-Gel TSK Phenyl-5-PW HPLC column equilibrated with 500 mM potassium phosphate buffer, pH 7.0, containing 10 mM MgCl₂ and 2 mM DTT. The column was washed at a flow rate of 0.75 ml/min until all of the unbound protein was removed. At this time, a 60-ml linear gradient of 500 mM to 0 mM K₂HPO₄ was applied, to the column utilizing 30 ml of 500 mM K₂HPO₄, pH 7.0, and 30 ml of lysis buffer without glycerol. Fractions (3 ml) were collected and assayed for PMI-GMP activity. The active fractions were pooled and concentrated to 5 ml by ultrafiltration.

**Step 3**—A Q-Sepharose column (1.5 × 30 cm) was equilibrated with lysis buffer, and the enzyme solution from Step 2 was applied to the column. After washing the column with 100 ml of lysis buffer, the bound proteins were eluted with a 200-ml 0 to 0.5 M NaCl gradient. Fractions of 3 ml were collected, assayed for PMI-GMP activity, and concentrated to 2 ml as described in Step 2 above.

**Step 4**—A Sephacryl S-200 gel filtration column (2.5 × 100 cm) was equilibrated with 100 mM MOPS buffer, pH 7.5, containing 100 mM NaCl, 10 mM MgCl₂, 15% glycerol, and 2 mM DTT. The enzyme solution from Step 3 was applied to the column, and fractions (3 ml) containing PMI-GMP activity were assessed for purity by SDS-PAGE according to the method of Laemmli (22). Gels were stained for protein using Coomassie Blue G-250. The N-terminal amino acid sequence of the purified protein was determined by Dr. Ka-Leung Ngai at Northwestern University, Chicago, IL.

**Determination of Native Molecular Weight**

The native molecular weight of PMI-GMP was determined by fast protein liquid chromatography gel filtration on a 1.0-× 30-cm Superose 6 column equilibrated with 100 mM MOPS buffer, pH 7.5, containing 200 mM NaCl. Carbonic anhydrase (Mₙ = 29,000), bovine serum albumin (Mₙ = 66,000), alcohol dehydrogenase (Mₙ = 150,000), and β-amylase (Mₙ = 200,000) served as molecular weight standards. A 100-μl aliquot of PMI-GMP from Step 3 was applied to the column, and 1-ml fractions were assayed for PMI-GMP activity.

**Identification of Reaction Products**

The PMI forward reaction mixture contained 100 mM MOPS buffer, pH 7.0, 1 mM CoCl₂, 1 mM F6P, and 2 μCi/ml [d-¹⁴C] fructose 1,6-bisphosphate (250,000 cpm/mmol). The PMI reverse reaction contained 100 mM MOPS buffer, pH 7.0, 1 mM CoCl₂, 10 mM M6P, and 0.6 μCi/ml d-¹⁴C mannose 6-phosphate (1 mCi/mmol). The

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**TABLE I**

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<td>his-1, Alg⁺ (spontaneous derivative of 8821)</td>
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**Plasmids**

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PMI reactions contained 200 μg/ml purified enzyme and were carried out at 25 °C for 2.5 h. Reaction mixtures with boiled enzyme served as a control. In some cases, the reactions were terminated by boiling for 7.5 min and then treated with 1 unit/ml of an agarose bead-insoluble d-fructose-6-phosphate kinase, 4 mM ATP, 1 mM DTT, 50 mM β-glycerophosphate, and 2 mM EDTA at 37 °C for 5 h. Treatment with d-fructose-6-phosphate kinase converted F6P to d-fructose 1,6-diphosphate, thereby allowing better separation of the substrate and product.

The GMP forward reaction mixture contained 100 mM MOPS buffer, pH 7.5, 10 mM MgCl₂, 1 mM MgF₃, and 1 mM GTP. The radioactive substrates were 10 μCi/ml [8-³²]H]guanosine 5'-triphosphate (70 Ci/mmol), 10 μCi/ml guanosine 5'-α-[³²]P]triphosphate (410 Ci/mmol), 100 μCi/ml guanosine 5'-γ-[³²]P]triphosphate (5000 Ci/mmol), or 2.3 μCi/ml d-[U-¹⁴C]mannose 1-phosphate (254 mCi/mmol). The GMP reverse reaction mixture contained 100 mM MOPS buffer, pH 7.5, 10 mM MgCl₂, 1 mM GDP-M, 0.5 μCi/ml GDP-α-[³²]C]mannose (254 mCi/mmol), and 2 mM PPi. The GMP reactions contained 200 μg/ml purified enzyme and were performed at 25 °C for 15 min. Boiled enzyme or omission of substrate served as a control.

Reaction products were identified by thin layer chromatography performed at 37 °C (23, 24) on cellulose plates (solvent A: water (60:10:30); solvent B: methanol/ammonium hydroxide/water (80:15:5); solvent C: 95% ethanol/1 M ammonium acetate, pH 5.0 (7:3); solvent D: methanol/1 M ammonium hydroxide/water (60:10:30); solvent E: 1-propanol/ammonium hydroxide/water (60:30:10); solvent F: water-saturated phenol (25 °C) or on phosphothelylamine plates (solvent G: 2 M LiCl/2 M formic acid (1:1)). Carbohydrates were identified by staining with triphenyltetrazolium chloride (triphenyl tetrazolium chloride spray kit, Sigma) or alkaline peroxidase (23). Phosphosugars were stained with molybdate (24). Nucleotides and nucleosides were detected at 254 nm. ³²C-Labeled and ³²P-labeled compounds were detected by autoradiography on Kodak XAR.6 film. ³²P-Labeled compounds were detected by autoradiography on Amersham Hyperfilm-³²H.

Cloning and Sequencing of the algA Mutant Gene

The previously described algA sequence revealed that the entire open reading frame could be cloned as a 2.0-kb BamHI/SstI fragment (25). Isolated chromosomal DNA (26) from the P. aeruginosa Alg mutant 8822 was digested with BamHI/SstI, and fragments corresponding to 2 kb in size were electroeluted from a 0.7% agarose gel (27, 28). These fragments were ligated into the expression vector pMMB22 as described earlier for the wild-type algA gene (27) and characterized by restriction mapping using the wild-type algA sequence as a guide (28). Clones containing the mutant algA gene were mutagenized into the expression plasmid pCD1 (17). Sequencing of this mutant algA gene was accomplished by subcloning portions of the algA gene into Mpi1 and Pmp as a 200-bp HindIII/SphI fragment, a 1000-bp SphI/PstI fragment, or an 800-bp PstI/SalI fragment. This strategy permitted the sequencing of the entire 2.0 kb algA gene. Sequencing was accomplished by the Sanger dideoxy method using Sequenase as described earlier (29). Reactions were electrophoresed in either 3% acrylamide gradient gels or 4% non-gradient gels in order to clearly read >500 bp. The wild type sequence was run in parallel with the mutant sequence in order to detect nucleotide changes.

RESULTS

PMI-GMP Purification and Characterization—A major problem in the purification of alginate enzymes is that the specific activities of all the P. aeruginosa alginate biosynthetic enzymes are so low that the levels of PMI, phosphomannomutase, GMP, and GDP-M dehydrogenase border on the limits of detectability (13, 14). This makes the conventional method of enzyme purification from crude extracts extremely difficult. Table II shows that both PMI and GMP activities were not detectable in the mucoid strains 8821 and 8830, even though they produce copious amounts of alginate (18). Conjugation of the plasmid pAD4033 (Fig. 1), which contains the algA gene under control of the tac promoter (17), into P. aeruginosa strain 8822 resulted in the appearance of high levels of both PMI and GMP activities when induced with IPTG (Table II). When the E. coli PMI- strain CD1 was transformed with pAD4033 and grown in the presence of 1 mM IPTG, both PMI and GMP activities were easily detectable in crude extracts (Table II). GMP activity is normally not detectable in E. coli.

The purification of PMI-GMP as shown in Fig. 2 was initiated with hydrophobic interaction chromatography utilizing a Bio-Gel TSK Phenyl 5-PW HPLC column. Following fractionation of the crude extract, both PMI and GMP activities eluted from this column at the very end of a descending phosphate gradient (Fig. 2). After pooling the fractions containing both PMI and GMP activities, assays for each activity revealed a 10-fold and 11-fold increase in PMI and GMP specific activities, respectively. When this enzyme preparation was further fractionated via ion exchange chromatography utilizing Q-Sepharose, the PMI and GMP activities once again co-eluted. Subsequent gel filtration chromatography of

<table>
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<th>Table II</th>
<th>Specific activities of PMI and GMP in P. aeruginosa and E. coli</th>
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*The levels of <1 indicate the lower limits of detectability.  
The tac promoter is leaky in P. aeruginosa, but not in E. coli, giving low levels of PMI-GMP activity in the absence of IPTG only in P. aeruginosa.

FIG. 1. The P. aeruginosa alginate biosynthetic pathway and the organization of the gene cluster containing the algA gene. A, the proposed pathway of alginate synthesis. GDP-MAN, GDP-D-mannuronic acid. B, chromosomal organization of the alginate genes. The arrows indicate the direction of transcription for the algD gene (encoding GDP-M dehydrogenase) and the algA gene (encoding PMI-GMP). C, genetic map of plasmid pAD4033 (17) containing the algA gene under control of the tac promoter in the expression vector pMMB22 (19).
Phosphomannose Isomerase-Guanosine Diphospho-D-mannose Pyrophosphorylase

this Q-Sepharose preparation yielded a single major protein peak that retained both PMI and GMP activity (Fig. 2, Table III). The total purification for PMI and GMP activities were 14- and 15-fold, respectively (Table III). SDS-PAGE of the purified PMI-GMP revealed a single polypeptide with a molecular weight of 56,000 (Fig. 3). The native protein was estimated to be a monomer of 54,000 molecular weight. It is important to note that during the course of the purification of the enzyme to homogeneity the ratio of the specific activities of GMP and PMI remained constant. In addition, the N-terminal amino acid sequence determined from this protein exactly matched that predicted from the algA DNA sequence (Fig. 4).

Early attempts to purify PMI-GMP resulted in large losses in enzymatic activity due to cold sensitivity of the enzyme. The addition of 15% glycerol to all purification buffers stabilized the enzyme and improved enzymatic activity. However, the enzyme also tended to precipitate during concentration of column fractions. This problem was alleviated by adding NaCl to a final concentration of 200 mM whenever the enzyme was concentrated. Characterization of the purified PMI-GMP enzyme revealed that divalent metals are absolutely necessary for both PMI and GMP activity (Fig. 5). For PMI activity, the order of activation is Co²⁺ > Ni²⁺ > Mn²⁺ > Mg²⁺ > Ca²⁺ > Zn²⁺. However, the GMP reaction only utilized either Mg²⁺ or Mn²⁺ to convert M1P and GTP to GDP-M and PP₁ (Figs. 1 and 5).

The apparent Kₘ values for the PMI-GMP enzyme substrates were determined by double reciprocal plots (Fig. 6). Analysis of the PMI reaction revealed that the Kₘ and Vₘₐₓ values for M6P were 3.03 mM and 830 nmol/min/mg of enzyme, respectively. This assay was performed in the presence of 1 mM CoCl₂ at the optimum pH of 7.0. The Kₘ for F6P could not be determined, since there is no effective method to reliably quantitate M6P formation. Using radioactive substrates, we have determined that the PMI reaction will produce M6P from 1 mM F6P, whereas the reverse PMI reaction does not produce very much F6P even in the presence of 10 mM M6P (see Identification of PMI-GMP Reaction Products, below; Fig. 7A). However, this radioactive assay is not reliable enough to determine kinetic parameters for the PMI forward reaction.

The double reciprocal plot for the forward GMP reaction showed that the Kₘ for M1P was 20.5 μM in the presence of 1 mM GTP (Fig. 6), and the Kₘ for GTP was 29.5 μM in the presence of 1 mM M1P. Both plots intersected at the same Vₘₐₓ value, and the maximal velocity measured for the forward GMP reaction was 5680 nmol/min/mg of protein at the optimum pH of 7.6. This specific activity is similar to the value of 5170 nmol/min/mg of protein obtained for the reverse GMP reaction utilizing 1 mM concentration each of GDP-M and PP₁, at the optimum pH of 7.6 (Fig. 6). The Kₘ for GDP-M was determined to be 14.2 μM, but the Kₘ for PP₁ could not be accurately determined because the reaction did not obey linear kinetics (data not shown). However, we did determine that 1 mM PP₁ does not limit the rate of the GMP reverse reaction.

In an effort to evaluate the involvement of sulfhydryl groups in PMI-GMP activity, the purified enzyme was preincubated with DTT and then assayed for activity. The PMI activity showed a high sensitivity to this reducing agent, resulting in a 50% inactivation immediately after the addition of 0.1 mM DTT. The PMI activity was restored when the DTT was removed by dialysis. The GMP reaction was not affected by up to 5 mM DTT. These results suggest that some of the sulfhydryl groups must remain oxidized for maximal PMI.

![Figure 2](image)

**Fig. 2. Purification of PMI-GMP.** A, Bio-Gel TSK Phenyl-5PW chromatography of crude extract. The enzyme was applied to the column in 500 mM phosphate buffer and eluted with a step gradient utilizing 100 mM MOPS, pH 7.0. B, ion exchange chromatography of PMI-GMP active protein peak from A utilizing Q-Sepharose. The proteins were eluted with a linear 0 to 500 mM NaCl gradient. C, gel filtration chromatography of PMI-GMP active peak from B on Sephacryl-200.

### Table III

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<td>GMP</td>
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<td>GMP</td>
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<tr>
<td></td>
<td>mg</td>
<td>milliunits/mg</td>
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<td>14 15</td>
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PMI-GMP purification was described under “Experimental Procedures.” PMI activity was measured in the reverse direction, and the GMP rate is that for the forward reaction coupled to purified GDP-M dehydrogenase and NAD⁺ reduction.
activity, presumably as disulfide bonds, to create a protein structure capable of catalyzing the conversion of M6P to F6P. Despite the obvious sensitivity of this enzyme to DTT, enzyme stability during purification is maintained only when either DTT or β-mercaptoethanol is included in the purification buffers. The enzyme is routinely purified in the presence of 2 mM DTT, and the reducing agent is removed through dialysis before determining specific activity. Preincubation of PMI-GMP with a 10-fold excess of iodoacetate for various time periods did not result in a loss of either enzymatic activity. These results indicated that either the sulfhydryl groups of Cys may not play a direct role in catalysis or are not accessible to iodoacetate.

Several compounds were tested as alternative substrates and/or inhibitors of the PMI and GMP activities present in the purified enzyme. The PMI reaction could not convert d-glucose 6-phosphate to F6P, and nucleotides such as ATP, GTP, CTP, UTP, and TTP (or their respective mono- and dinucleotide phosphate derivatives) did not inhibit PMI activity (data not shown). The GMP reverse reaction could not utilize GDP-d-glucose, ADP-d-mannose, or UDP-d-mannose, nor would the corresponding glucose derivatives of these dinucleotides serve as a substrate. Of particular interest was the lack of an inhibitory effect of the products and substrates of the GMP reaction on the PMI reaction and vice versa. For example, neither M6P nor F6P inhibited GMP activity, and the addition of GTP, M1P, GDP-M, or PP, either individually or together did not affect PMI activity. This indicates that the substrate binding and/or active sites for the PMI and GMP activities do not share common domains of the polypeptide.

Identification of PMI-GMP Reaction Products—The PMI enzymatic activity is thought to catalyze the reversible conversion of F6P to M6P (Fig. 1). An earlier study by Gill et al. (17) demonstrated that a crude extract containing the hyper-produced algA gene product could convert F6P to a product which co-migrated with M6P. The successful purification of PMI-GMP in this study has enabled us to analyze the PMI and GMP reactions under more favorable conditions utilizing pure enzyme. Incubation of the enzyme with [14C]F6P yielded a radioactive spot which co-migrated with M6P (solvents A, B, C, G). Although the substrate and product were separated in solvent A (Fig. 7A), conversion of the remaining [14C]F6P to D-fructose 1,6-diphosphate via D-fructose-6-phosphate kinase provided better separation and allowed easy identification of the M6P spot as the product of the PMI forward reaction (Fig. 7C). The putative M6P spot was eluted from the plate and treated with alkaline phosphatase (15), yielding a radioactive spot which co-migrated with d-mannose in solvents E and F. In addition, treatment of the product of the PMI forward reaction with boiling in 0.1 N HCl for 10 min did not release d-mannose, indicating that the phosphate linkage was most likely linked stably to carbon-6 rather than carbon-1 of d-mannose (30). Incubation of PMI-GMP with as high as 10 mM M6P yielded only a small amount of product which could be converted to d-[14C]fructose 1,6-diphosphate through the addition of d-fructose-6-phosphate kinase (Fig. 7, B and D). This provided evidence that F6P was the product of a slow reverse PMI enzymatic activity.

The GMP enzymatic activity is thought to catalyze the reversible synthesis of GDP-M and PP, from M1P and GTP (Fig. 1). Incubation of the enzyme with [3H]GTP and unlabeled M1P yielded a radioactive and fluorescent spot which co-migrated (solvents E and F) with GDP-[14C]mannose and thereby indicated that this phosphodiesterase yielded either a radioactive guanosine spot (solvents E and F) as expected for [3H]GDP-M or a radioactive spot co-migrating (solvents E and F) with d-mannose as expected for GDP-[14C]mannose. Boiling the GMP forward reaction product in acid released either d-[14C]mannose from putative GDP-[14C]mannose or [3H]GDP and [3H]GMP from putative [3H]GDP-M. The addition of [α-32P]GTP also resulted in a radioactive spot which co-migrated (solvents B, C, E, and G) with GDP-M (Fig. 7G), whereas incubation of the enzyme with [γ-32P]GTP yielded a radionabeled spot which co-migrated (solvents A, B, C, E, and G) with PP (Fig. 7H).

GMP activity in the reverse direction was monitored by adding GDP-[14C]mannose. The radioactive product co-migrated (solvents A, C, D, and E) with M1P (Fig. 7F) and was cleaved to d-mannose either by treatment with alkaline phosphatase or by boiling in 0.1 N HCl. These results identified the GMP reverse reaction product as M1P on the basis of co-chromatography with authentic M1P, dephosphorylation by alkaline phosphatase, and acid lability of the phosphate group (indicating a carbon-1-linked phosphate group (30)). In combination, these results showed that the products of the reversible PMI and GMP enzymatic activities were the same as those proposed in Fig. 1.

Cloning and Expression of a Mutant algA Gene—An earlier study by Darzins et al. (25) demonstrated that disruption of the algA gene with a kanamycin cassette, creating plasmid pAD4040, resulted in a complete loss of PMI activity. Table IV shows that crude extracts prepared from IPTG-induced P. aeruginosa/pAD4040 lack both PMI and GMP activities, indicating that algA encodes both activities. The nonmucoid P. aeruginosa strain 8853 is complemented to mucoidy when the plasmid pAD4033 is present (18). This indicated that the inability of this strain to produce alginate was due to a mutation in the algA gene. In order to determine the nature of this defect, we cloned and sequenced the mutant algA gene from 8853 using the protocol described under “Experimental Procedures.” Cloning the defective algA gene into pMMB22.
which has both phosphomannose isomerase and GDP-D-mannose pyrophosphorylase activities. PMI-GMP is a 54,000 molecular weight protein which migrates as a single 56,000 molecular weight protein which migrates as a single 56,000

**Discussion**

In this study we have purified the algA gene product from P. aeruginosa and determined that it is a bifunctional enzyme which has both phosphomannose isomerase and GDP-D-mannose pyrophosphorylase activities. PMI-GMP is a 54,000 molecular weight protein which migrates as a single 56,000 molecular weight polypeptide in a SDS-PAGE system. This size is close to the value of 53,134 calculated from the amino acid composition of the protein. N-terminal amino acid sequence analysis of the pure protein exactly matched the algA sequence, confirming that PMI-GMP is the product of the algA gene.

Evidence for bifunctionality was first obtained by confirming the work of Sá-Correia et al. (14) which showed that IPTG induction of algA in E. coli led to the simultaneous appearance of low PMI and GMP activities in cell extracts. It is also important to note that sequence analysis of the BamHI-SstI fragment containing the algA gene reveals a single open reading frame initiating at nucleotide 961, showing a G to A point mutation which results in a Val-322 to Met change in the amino acid sequence. The boxed, indicated at nucleotide position 961, showing a 10 amino acids are present in the nucleotide sequence (25). As a result, we created pAB8853, which is essentially the same plasmid depicted in Fig. 1 except that the wild-type algA gene has been replaced by the mutant 8858 gene. Conjugation of pAB8853 into P. aeruginosa strain 8822 demonstrated that IPTG induction led to the appearance of low PMI and GMP activities (Table IV). This mutant PMI-GMP enzyme produced only 23% and 6% of the wild-type PMI and GMP activities, respectively, indicating that the mutation in this gene had affected a region of the protein critical for both enzymatic activities. Sequencing of this mutant algA gene revealed that a single base change at nucleotide 961 resulted in the replacement of valine with methionine (Fig. 4).
have included the correct sequence in this manuscript (Fig. 4).

There are several reports citing the purification and character-
ization of bifunctional enzymes, and much of the work has cen-
tered on the tryptophan biosynthetic pathway. Bifunc-
tional enzymes such as the E. coli chorismate mutase-
prephenate dehydratase (31) and N-(5'-phosphoribo-
syl)anthranilate isomerase-indole-3'-glycerol phosphate syn-
thatase (32) catalyze sequential reactions involving the con-
versatraction of structurally similar substrates. However, there are
enzymes such as 3-deoxy-D-arabino-heptulosonate 7-phos-
phate synthetase-chorismate mutase from Bacillus subtilis
which carry out noncontiguous reactions involving structur-
dissimilar substrates (33). PMI-GMP carries out reac-
tions 1 and 3 of alginate biosynthesis utilizing substrates
which are very different structurally. For instance, the PMI
reaction involves the reversible isomerization of F6P to M6P
while the GMP activity catalyzes the charging of a sugar
phosphate with the nucleotide CTP. These two reactions are
very different mechanistically and do not appear to share
phosphate with the nucleotide GTP. These two reactions are
common substrate binding sites. This was shown by the lack
of activity present for either PMI or GMP in the absence of a metal cofactor, and only those metals which activated the enzyme are presented in this figure.

Control reactions demonstrated that the coupling enzymes (phospho-
glucose isomerase and glucose-6-phosphate dehydrogenase for PMI; GDP-M dehydrogenase for GMP) were active in the absence of metal cofactors.

In this study we found no inhibition of either PMI or GMP
by possible alginate biosynthesis intermediates such as D-
mannuronic acid or guanosine 5'-monophosphate. The latter
compound could be produced when the GDP-D-mannuronic
acid produced by the GDP-M dehydrogenase reaction is
cleaved in a subsequent polymerization reaction to guanosine
5'-monophosphate and some phosphorylated form of D-mann-
uronic acid (15). Interestingly, guanosine 5'-monophosphate
competitively inhibits GDP-M dehydrogenase with a K_i
of 22.7 μM (15), indicating that a mechanism of feedback inhi-
bition may exist to regulate alginate synthesis in P. aerugi-
 nosa.

PMI enzymes have been isolated from muscle (20, 35) and
yeast (36), and the K_m of the yeast enzyme for M6P was
determined to be 0.8 mM. In this study, we have determined
that the PMI-GMP of P. aeruginosa has a relatively high K_m
of 3.03 mM for M6P, indicating that the reverse PMI reaction
involving the conversion of M6P to F6P may not proceed very
efficiently in P. aeruginosa. An earlier study by Darzins et al.
(16) showed that a plasmid carrying the algA gene could
restore colanic acid (D-glucuronic acid:D-galactose:L-fucose,1:2:1:2 (39)) synthesis in the E. coli mutant
CD1. (Colanic acid synthesis requires the forward PMI reac-
tion to convert F6P to M6P.) However, the presence of this
plasmid would not permit CD1 to utilize D-mannose as a sole
source of carbon, an ability which requires the reverse PMI
reaction. The E. coli manA gene encoding PMI (required
for colanic acid synthesis and growth on D-mannose) has been
cloned and sequenced by Miles and Guest (37), but there have
not been any reports regarding enzyme isolation and charac-
terization. At this point we can surmise that the E. coli PMI
is utilized for polysaccharide (colanic acid) synthesis and for
the conversion of M6P to F6P when E. coli is utilizing D-
mannonse as a sole source of carbon. For the *P. aeruginosa* PMI-GMP, the high $K_m$ value for M6P indicates that the PMI reaction highly favors M6P formation and therefore is suited well for alginate production but not for d-mannonse utilization.

The metal specificity for the GMP reaction is limited to Mg$^{2+}$ and Mn$^{2+}$, whereas the PMI reaction can utilize a variety of divalent metals. It is interesting to note that the PMI reaction rate with the GMP preferred metal, Mg$^{2+}$, is only 25% of the rate attained with Co$^{2+}$. This raises the question of which metal cofactor(s) is utilized in the cell to catalyze the independent PMI and GMP reactions. The PMI of red blood cells is a metal-dependent enzyme which prefers Mg$^{2+}$ over Co$^{2+}$ (38), and the GMP enzyme of both yeast (36) and the bacterium *Arthrobacter* sp. NRRL B1973 (30) has an absolute requirement for Mg$^{2+}$. Therefore, it appears that PMI-GMP, like all of the other GMP enzymes studied thus far, has an obligate requirement for a metal cofactor.

PMI-GMP was found to catalyze the predicted reactions in the alginate biosynthetic pathway described in Fig. 1. The identification of the reaction products was certain since the product of the forward reactions could be demonstrated to be the substrate for the reverse reactions and vice versa. In addition, the products could be coupled to other known enzymatic reactions. For instance, the reverse PMI assay depends upon the formation of F6P which is then coupled to NADP$^+$ reduction via phosphoglucose isomerase and d-glucose-6-phosphate dehydrogenase. Likewise, the reverse GMP assay depends upon the production of GTP which serves as a substrate for nucleotide-5-diphosphate kinase as described under “Experimental Procedures.”

Incubation of PMI-GMP with radioactive F6P or M6P showed that the equilibrium of the PMI reaction favors M6P formation. In addition, the kinetic data indicate a high $K_m$ for M6P. It has been reported by our laboratory that overexpression of PMI-GMP leads to an increase in phosphomannomutase activity (14). The kinetic data for the PMI reaction suggests that high PMI activity could lead to the accumulation of M6P, which then induces phosphomannomutase and leads to the observed increase in enzymatic activity. This hypothesis is supported by data showing an increase in phosphomannomutase activity when the *E. coli* PMI enzyme is expressed in *P. aeruginosa* (14). The determination of the mechanism for phosphomannomutase induction is currently under study in our laboratory.

The GMP reaction was shown to be responsible for the formation of the first nucleotide sugar of the alginate biosynthetic pathway, GDP-D-mannose. Utilizing the procedure developed by Roychoudhury et al. (15), we were able to purify the alginate biosynthetic enzyme GDP-M dehydrogenase (Fig. 1) from *P. aeruginosa* and use it to pull the GMP reaction forward as a coupled assay system. The GMP forward reaction rate was measured this way, and the $K_m$ values of 20.5 and 29.5 μM for M1P and GTP were obtained utilizing this coupled assay. In their study of the alginate biosynthetic enzyme GDP-M dehydrogenase, Roychoudhury et al. (15) determined the $K_m$ for GDP-D-mannose to be 14.9 μM. Since the enzymatic activity of the alginate biosynthetic enzymes is inherently low, we expected the enzymes to have comparable $K_m$ values for their substrates, and this study shows that this is indeed the case for GMP and GDP-M dehydrogenase.

Further evidence supporting the bifunctionality of the algA gene product was obtained by cloning the algA gene from the *P. aeruginosa* Alg$^-$ strain 8853. DNA sequencing showed that a single G to A base change at nucleotide 961 replaces Val-
321 with Met, resulting in a dramatic decrease in both PMI and GMP enzymatic activities (Table IV). These low activities prevent strain 8853 from synthesizing alginate; therefore, the colonies are non-mucoid on solid media. The large decrease in both enzymatic activities as a result of this point mutation shows that there is at least one region of the polypeptide which is important for both enzymatic activities. However, it is possible that the longer side chain of Met severely disrupts the protein conformation, resulting in a large decrease in both enzymatic activities. We are currently determining the importance of Val-322 by substitution with Ala via site directed mutagenesis. In addition, we are also analyzing other P. aeruginosa mutant algA genes in an effort to determine whether there are independent domains of the polypeptide that are responsible for either PMI or GMP activity.

The DTT sensitivity of the PMI reaction (but not GMP) suggests that disulfide bonds are necessary for the formation of the active site and/or substrate binding. The development of a successful purification protocol from this study will allow us to determine biochemically how many of the 8 Cys residues present in this protein may participate in disulfide bond formation. In addition, site-directed mutagenesis which substitutes Ala for Cys residues may reveal which Cys are necessary for a protein conformation which is conduсtive to PMI catalysis. Homology searches at the amino level did not reveal any significant areas of homology with the PMI of E. coli, and at this point the sequencing and characterization of mutant algA genes that we currently have is vital to obtaining mutants which lack only one of the activities. The future biochemical, genetic, and crystallographic studies of this unique bifunctional enzyme should further our understanding of alginate biosynthesis and provide us with the opportunity to determine if the PMM enzyme interacts directly with PMI-GMP in order to provide the channeling of F6P to GDP-M, thereby overcoming the constraints of extremely low levels of these enzymes.

REFERENCES