Identification of Amino Acid Residues Involved in the Activity of Phosphomannose Isomerase-Guanosine 5'-Diphospho-D-mannose Pyrophosphorylase

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

(Received for publication, March 23, 1993, and in revised form, September 23, 1993)

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Phosphomannose isomerase-guanosine 5'-diphospho-D-mannose pyrophosphorylase (PMI-GMP), which is encoded by the algA gene, catalyzes two noncontiguous steps in the alginate biosynthetic pathway of Pseudomonas aeruginosa: the isomerization of 1-fructose 6-phosphate to D-mannose 6-phosphate and the synthesis of GDP-D-mannose and PPI from GTP and D-mannose 1-phosphate. Amino acids that are required for the GMP enzyme activity were identified through site-directed mutagenesis of the algA gene. Mutation of Lys-175 to arginine, glutamine, or glutamate produced an enzyme whose $K_m$ for D-mannose 1-phosphate was 470-3,200-fold greater than that measured for the wild type enzyme. In addition, these mutant enzymes had a lower $V_{max}$ for the GMP activity as compared with the wild type PMI-GMP. These results indicate that Lys-175 is primarily involved in the binding of the substrate D-mannose 1-phosphate, although it is likely that other residues are required for the specificity of binding. Mutation of Arg-19 to glutamine, histidine, or leucine resulted in a 2-fold lower $V_{max}$ for the GMP enzyme activity and a 4-7-fold increase in the $K_m$ for GTP as compared with the wild type enzyme. Thus, it appears that Arg-19 functions in the binding of GTP. In addition, chymotryptic digestion of PMI-GMP showed that the carboxyl terminus is critical for PMI activity but not for GMP activity. Taken together, these results support the hypothesis that the bifunctional PMI-GMP protein is composed of two independent enzymatic domains.

Pseudomonas aeruginosa causes severe and debilitating pulmonary infections of children and young adults afflicted by CF 

(1). The abnormal exocrine mucous secretions present in the lungs of these patients induce P. aeruginosa to produce a vis-

ous exopolysaccharide called alginate (2). Alginic acid of mucoid P. aeruginosa is a linear copolymer composed of 1-4-linked D-mannuronic acid (partially O-acetylated) and the C-5 epimer L-guluronic acid (3-5). Alginic acid of mucoid P. aeruginosa infects the lungs of CF patients (1). Elimination of the alginate barrier would likely enhance treatment of chronic pulmonary infections by P. aeruginosa and improve the prognosis for CF patients. Thus, we are studying the biochemistry and genetics of the alginate pathway in P. aeruginosa in an effort to identify nontoxic inhibitors of alginate biosynthetic enzymes.

Mucoid strains of P. aeruginosa produce low levels of the alginate biosynthetic enzymes (Fig. 1), whereas these enzymes are either absent or greatly reduced in nonmucoid strains (6, 7). Many of the alginate biosynthetic genes have been cloned and sequenced (8-13). In addition, cloning alginate genes under control of a strong promoter (e.g. tac) has been used to produce sufficient amounts of their respective gene products for identification of enzyme function (9, 11-14, 16, 17). This strategy also facilitated the purification of PMI-GMP (16), a bifunctional enzyme that catalyzes the first and third steps of alginate biosynthesis, and GDP-5-mannose dehydrogenase (17), which catalyzes the fourth step of the alginate pathway of P. aeruginosa (Fig. 1).

The purified PMI-GMP protein exhibits a number of characteristics that suggest that the PMI and GMP enzymatic activities may reside in separate catalytic domains (16). First, PMI activity (but not GMP activity) is inhibited by sulfhydryl reagents. Second, GMP activity is dependent on the presence of either Mg$^{2+}$ or Mn$^{2+}$, whereas the PMI reaction utilizes a variety of divalent metals with Co$^{2+}$ giving maximal activity. Third, the substrates and products of the GMP reaction do not inhibit the PMI enzyme activity and vice versa. Fourth, there are large structural differences between the substrates for PMI and GMP as well as mechanistic differences between an isomerization reaction and the charging of sugar phosphate with a nucleotide. In this study, we used site-directed mutagenesis and limited proteolysis to identify regions of the algA-encoded protein necessary for the PMI and GMP enzymatic activities.

EXPERIMENTAL PROCEDURES

Materials—SDS, acrylamide, bisacrylamide, Coomassie Blue G-250, Bradford protein assay reagent, and the Bio-Gel TSK phenyl-5PW HPLC column were from Bio-Rad. All other chemicals were obtained from Sigma unless indicated otherwise. Phosphoglucone isomerase (EC 5.3.1.9), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), and hexoki-

nase (EC 2.7.1.1) were also purchased from Sigma. The Q-Sepharose resin and Mono-Q column were obtained from Pharmacia 1KB Biotechnology, Inc.
Strains and Plasmids—The bacterial strains and plasmids used in this study are shown in Table I. P. aeruginosa 8830 is an alginate-producing (Alg') strain isolated from the spum of a CF patient, and, like other mucoid P. aeruginosa strains, spontaneously reverts to the nonmucoid (Alg-) form (18). The stable Alg' strain 8830 was obtained by chemical mutagenesis of the spontaneous nonmucoid strain 8822. The algA mutant strain 8853 was isolated after further mutagenesis of strain 8830 (18). The plasmid pDS1 (Fig. 2), which was used in directed mutagenesis of the algA gene, was constructed by cloning a 2-kilobase BamHI fragment, containing the algA gene, from pES119 (12) into pUC119 using standard methods (19).

Site-directed Mutagenesis of algA—Mutations in the algA gene were obtained using the oligonucleotide-directed-in vitro mutagenesis system of Amersham (version 2) except that pUC119 was used as the cloning vector. Single-stranded DNA of the pUC119 algA derivative, pDS1, was obtained using the helper phage M13K07 (Promega). Mutant oligonucleotide primers were prepared by Operon Technology, Inc. (Alameda, CA). Modifications in the algA gene were confirmed by DNA sequencing using a dideoxynucleotide chain termination method (20) with the Sequenase DNA polymerase (U. S. Biochemical Corp.) was used at 37 °C according to the directions of the manufacturer, and 7-deaza-dGTP was substituted for dGTP for all sequencing reactions to reduce compression artifacts (21). Preparation of sequencing gels, conditions of electrophoresis, and autoradiography were performed as described previously (22).

Each of the algA mutant genes was cloned as a 2-kilobase BamHI fragment into the BamHI site of the broad host range expression vector pMBB66HE using standard molecular biology techniques (19). The recombinant expression plasmids (Table I) were then mated into the algA gene of the wild type strain 8853 by triparental mating (23). Transconjugants were selected for on Pseudomonas isolation agar (Difco) containing 450 μg/ml carbenicillin. The transconjugants were then screened for the ability to produce alginate (i.e. complement strain 8853) by adding 1 mM IPTG to the medium and observing colonies for a mucoid phenotype.

The algA gene in P. aeruginosa 8853 was cloned in pUC119, by direct cloning into the BamHI site. The clones were grown in 500 ml of Luria 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 13,000 g for 15 min, washed once with 0.9% NaCl, and resuspended in 16 ml of lysis buffer (100 mM Tris-HCl, pH 7.0, 10 mM MgCl₂, 15% glycerol, 2 mM dithiothreitol). The cells were disrupted by sonic vibration in an oscillator (Branson) and centrifuged at 40,000 g for 15 min. The resulting supernatants were then centrifuged at 100,000 g for 1 h, and the clarified supernatants were used for PMI-GMP purification. PMI-GMP was purified using a Q-Sepharose column and a Bio-Gel TSK phenyl-5-PW HPLC column according to procedures published previously (16) except that MOPS buffer was replaced by 100 mM Tris-HCl, pH 7.0. Fractions containing PMI-GMP activity were assessed for purity by SDS-PAGE and staining with Coomassie Blue G-250 (24). The wild type and mutant proteins were estimated to be greater than 95% pure.

PMI and GMP Assays—PMI activity was assayed in the reverse direction by the method of Stein (25) using a modified assay mixture (16). The rate of NADP⁺ reduction was monitored at 340 nm at 25 °C in a Gilford Response II spectrophotometer. GMP activity was assayed in the forward direction by coupling the reaction to GDP-β-mannose dehydrogenase as described by Shinabarger et al. (16). The rate of NADP⁺ reduction was monitored at 340 nm at 25 °C.

Protocols and Procedures—Purified PMI-GMP (5 mg) was incubated with 250 μg of chymotrypsin for 45 min at 25 °C in the presence of 1 mM D-mannose-1-phosphate, 1 mM GTP, and 10 mM MgCl₂. The GMP substrates were found to increase greatly the stability of the major chymotryptic fragment. The chymotryptic reaction was then treated with 250 μg of trypsin/chymotrypsin inhibitor and subsequently dialyzed 2 volumes of 100 mM Tris-HCl, pH 7.0, containing 10 mM MgCl₂, 2 mM dithiothreitol, and 15% glycerol. The sample was loaded onto a Mono-Q column (Pharmacia) that had been equilibrated in the same buffer. Protein was eluted with a 60-ml linear gradient of 0-250 mM NaCl at a flow rate of 1 ml/min. The column fractions containing the 52-kDa chymotryptic fragment were pooled, concentrated using a Centricon-10 filter (Amicon), and then digested a second time with trypsin. The purified chymotryptic fragment was then assayed for PMI and GMP activity as described above. The amino-terminal amino acid sequence was determined by Dr. Ka-Leung Ngai at the University of Illinois at Urbana.

RESULTS AND DISCUSSION

Amino Acid Sequence Comparisons of PMI-GMP with Related Proteins—The amino acid sequence of the algA-encoded PMI-GMP was first compared with the sequences of proteins that catalyze similar enzymatic activities in E. coli. Two regions of PMI-GMP were, however, found to be quite similar to amino acid sequence similarity with the GMP enzymes RfbM and CpsB of S. typhimurium and E. coli. The XanB protein of Xanthomonas campestris (33), a bifunctional PMI-GMP enzyme involved in xanthan gum synthesis, was also found to be highly related to PMI-GMP of the alginate biosynthetic pathway (Fig. 3).

A comparison of PMI-GMP with other isomerases and pyrophosphorylases revealed a weak homology (23% identity) with the bacterial enzymes CDP-glucose pyrophosphorylase (29), UDP-glucose pyrophosphorylase (29, 34, 35) and ADP-glucose pyrophosphorylase (36, 37). Two regions of PMI-GMP were, however, found to be quite similar to ADP-glucose pyrophosphorylase. First, the Lys-175 region (FVEK) of PMI-GMP is identical to the substrate binding site of bacterial ADP-glucose pyrophosphorylase (36, 37). The enzymatic activity of ADP-glucose pyrophosphorylase (36, 37) also has remnants of this substrate binding site. This suggests that the amino acid sequence FVEK may be a substrate binding motif for this class of pyrophosphorylases. Second, the Lys-20 region of PMI-GMP is similar to the allosteric site of ADP-glucose pyrophosphorylase. Nonetheless, Lys-20, the putative allosteric residue according to the homology with ADP-glucose pyrophosphorylase, is not conserved among the GMP class of proteins (Fig. 3).

Oligonucleotide-directed-in Vitro Mutagenesis of the algA Gene—Fifteen independent mutations were made in the algA gene to change amino acids within the highly conserved regions of PMI-GMP (Fig. 3). The mutant genes were then cloned under control of the IPTG inducible tac promoter of the bacterial enzymes CDP-glucose pyrophosphorylase (29), UDP-glucose pyrophosphorylase (29, 34, 35) and ADP-glucose pyrophosphorylase (36, 37). Two regions of PMI-GMP were, however, found to be quite similar to ADP-glucose pyrophosphorylase. First, the Lys-175 region (FVEK) of PMI-GMP is identical to the substrate binding site of bacterial ADP-glucose pyrophosphorylase (36, 37). The enzymatic activity of ADP-glucose pyrophosphorylase (36, 37) also has remnants of this substrate binding site. This suggests that the amino acid sequence FVEK may be a substrate binding motif for this class of pyrophosphorylases. Second, the Lys-20 region of PMI-GMP is similar to the allosteric site of ADP-glucose pyrophosphorylase. Nonetheless, Lys-20, the putative allosteric residue according to the homology with ADP-glucose pyrophosphorylase, is not conserved among the GMP class of proteins (Fig. 3).
cloning the cated polypeptide is unstable and is subject to degradation. from the plasmid PES119 (12) into the BamHI site of pUC119. See "Experimental Procedures" for cloning details.

Transconjugants containing eight of the mutant plasmids contained the Alg- defect of strain 8853 in the presence of IPTG because the \( \text{algA} \) gene (BamHI-SstI) as a 2-kilobase BamHI fragment from the plasmid pES119 (12) into the BamHI site of pUC119. See "Experimental Procedures" for cloning details.

Mutation of Lys-175 to arginine, glutamine, or glutamate reduced the \( V_{\text{max}} \) for the GMP reaction compared with that of the wild type enzyme, where the \( V_{\text{max}} \) of the mutant was found to decrease with increasingly acidic amino acid replacements (Table II). These amino acid substitutions also increased the \( K_m \) for \( \text{d-mannose} \) 1-phosphate 470–3,200-fold over that observed for wild type PMI-GMP. PMI activity, however, was relatively unaffected by these mutational changes. These results indicate that the primary role of Lys-175 is to bind the GMP sugar (38). Although the size, shape, and charge of the amino acid are also important for proper substrate binding, the observed increase in the \( K_m \) for \( \text{d-mannose} \) 1-phosphate via an ionic interaction of the \( \epsilon \)-amino group of lysine with the negatively charged phosphate rather than with a hydroxyl group of the sugar (38). Although the size, shape, and charge of the amino acid are also important for proper substrate binding, the observed increase in the \( K_m \) for \( \text{d-mannose} \) 1-phosphate via an ionic interaction of the \( \epsilon \)-amino group of lysine with the negatively charged phosphate rather than with a hydroxyl group of the sugar (38).

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Hill et al. (38) found that mutational replacement of Lys-195 affected the kinetics of the \( \text{E. coli} \) ADP-glucose pyrophosphorylase reaction in a manner similar to that of the PMI-GMP Lys-175 mutations. The similarity to ADP-glucose pyrophosphorylase suggests that the PMI-GMP Lys-175 mutants might also have a higher \( K_m \) for \( \text{d-mannose} \) although the kinetics of the GMP reaction were not determined in the direction of pyrophosphorylation. ADP-glucose pyrophosphorylase is thought to bind its substrate \( \text{d-glucose} \) 1-phosphate via an ionic interaction of the \( \epsilon \)-amino group of lysine with the negatively charged phosphate rather than with a hydroxyl group of the sugar (38). Although the size, shape, and charge of the amino acid are also important for proper substrate binding, the observed increase in the \( K_m \) for \( \text{d-glucose} \) 1-phosphate as Lys-195 was replaced with increasingly acidic residues supports the view that lysine binding to phosphate is the basis for optimal binding affinity. The importance of Lys-175 in the PMI-GMP and Lys-195 of ADP-glucose pyrophosphorylase for phosphate binding is further supported by the occurrence of the same phosphate binding sequence (FVEKP) in several other pyrophosphorylase enzymes. Thus, it is not surprising that ADP-glucose pyrophosphorylase and PMI-GMP share a common binding sequence for the phosphate group yet bind different sugar phosphates. Additional amino acid residues are, however, likely required for the specificity of binding that is observed for both these enzymes (16, 38).

We were surprised that the \( K_m \) for \( \text{d-mannose} \) 1-phosphate...
ever, the negative charge of glutamate could also be neutralized, which could be different. This difference may allow glutamate to function in a way that is different from ADP-lysine (and to a lesser extent with an arginine residue). However, it is unclear exactly which amino acids are involved in the interaction between Lys-175 and this site. The use of azido-GTP should prove useful in identifying the guanine binding site and in elucidating any interaction between Lys-175 and this site.

The efficiency of CTP binding was also slightly reduced (2-3-fold) by replacing Lys-175 with arginine, glutamine, or glutamate (Table II). It is quite possible that mutation of Lys-175 changes the conformation of the phosphate binding site which in turn affects the binding of GTP. Alternatively, this result may indicate that Lys-175 participates in the binding of GTP to the guanine binding site. PMI-GMP lacks a consensus GTP binding site (41), so it is unclear exactly which amino acids are involved in guanine binding. In contrast to PMI-GMP, the Lys-195 mutants of ADP-glucose pyrophosphorylase exhibit wild type levels of ATP binding (38). Tyr-114 has been shown to be important for the binding of the adenine moiety of AMP, ATP, and ADP-glucose (40, 42-44). Although Lys-195 also binds AMP and ADP-glucose, ATP binds Tyr-114 in a manner that is thought to preclude an interaction of the α-phosphate oxygen with Lys-195 (38). If Lys-175 also has a role in GTP binding, then the guanine binding site of PMI-GMP would likely be oriented such that the α-phosphate oxygen of GTP can interact with Lys-175. The use of azido-GTP should prove useful in identifying the guanine binding site and in elucidating any interaction between Lys-175 and this site.

Mutation of Lys-20 to glutamine produced a protein that was unable to support alginate synthesis, yet kinetic analysis of the mutant enzyme failed to show any obvious reason as to why the protein is less efficient than the wild type enzyme (Table II). It is possible that the Lys-20 change affects the forward reaction by increasing the $K_m$ for n-fructose 6-phosphate, but we are unfortunately unable to measure accurately the kinetic constants for the n-fructose 6-phosphate to n-mannose 6-phosphate isomerization reaction. However, mutation of the neighboring Arg-19 to histidine, lysine, or leucine had a dramatic effect on GMP enzyme activity. These mutations increased the $K_m$ for GTP 4-7-fold, increased the $K_m$ for n-mannose 1-phosphate 2-8-fold, and decreased the $V_{max}$ for the GMP reaction 2-fold as compared with the wild type enzyme (Table II). PMI activity was relatively unaffected by these Arg-19 mutations.

Gardiol and Preiss (45) found that Lys-39 mutants of ADP-glucose pyrophosphorylase behaved similarly to the Arg-19 PMI-GMP mutants: a lower apparent affinity for the nucleotide triphosphate (4-fold) and a reduced binding affinity for the sugar phosphate (6-fold) in the presence of the activators. This suggests that Arg-19 of PMI-GMP may play a role in the allosteric regulation of GMP activity, but it should be noted that we have never observed an allosteric effect for PMI-GMP in vitro. However, it is plausible that the allosteric effect(s) is present in high enough concentrations in our assay system (e.g. one of the intermediates of the coupling enzymes or a contaminant of one of the substrates) so that the wild type PMI-GMP is fully activated in vitro. Alternatively, there is emerging evidence that activator binding by ADP-glucose pyrophosphorylase brings the glycine-rich region (analogous to the nucleotide binding P-loop motif) closer to Tyr-114 for more efficient binding of the nucleotide triphosphate (46). Thus, Arg-19 may be part of the GTP binding site involving glycine residues near Ser-10 (Fig. 3). Indeed, there seems to be a big effect of mutating Arg-19 on the $K_m$ of GTP. This site in PMI-GMP may not

with an uncharged amino acid (e.g. glutamine) abolishes phosphate binding, leading to a higher $K_m$ for the sugar phosphate substrate of both proteins. Although we favor this hypothesis, we cannot entirely rule out that conformational changes account for at least some of the differences between the wild type and Lys-175 mutant proteins. Determination of the crystal structure will allow us to examine further the microenvironment of the phosphate binding site, the effect of the mutations on the binding pocket, and the potential role of metal coordination in phosphate binding. Nonetheless, Lys-175 clearly participates in the binding of n-mannose 1-phosphate.

FIG. 3. Amino acid sequence comparison of the AlgA protein (PMI-GMP) from P. aeruginosa with the GMP proteins RfbM (29) and CpsB (30) from S. typhimurium and the PMI-GMP protein XanB (33) from X. campestris. Boxed sequences indicate exact identity of RfbM (49% identity), CpsB (53.4% identity), and XanB (59% identity) with the AlgA protein. Similarities with AlgA are greater than 83% when including conservative amino acid replacements. The aligned sequences depict entire proteins except for the first 3, 8, and 4 amino acids of RfbM, CpsB, and XanB, respectively. Two regions of AlgA, indicated by black boxes below the alignment, are similar to the allosteric (44% identity) and active (100% identity) sites of GlgC, the bacterial ADP-glucose pyrophosphorylase (36). * amino acids of the AlgA protein that were targeted by mutagenesis and are required for in vitro. The Val-321 change was obtained by cloning the defective gene from strain 4875 (16).

did not consistently increase as Lys-175 was changed from a basic to a neutral to an acidic amino acid, especially since n-glucose 1-phosphate binding by the ADP-glucose pyrophosphorylase Lys-195 mutants followed this trend (38). ADP-glucose pyrophosphorylase has a preponderance of proline and basic amino acids downstream of Lys-195 which have been implicated as part of an exposed substrate binding loop (37, 39, 40). PMI-GMP, however, contains fewer prolines and basic amino acids outside the immediate substrate binding site. Thus, it seems that the microenvironment of the phosphate binding sites of PMI-GMP and ADP-glucose pyrophosphorylase could be different. This difference may allow glutamate to function (albeit less efficiently than lysine) for GMP activity, whereas the phosphate binding pocket of ADP-glucose pyrophosphorylase has a more stringent requirement for a positively charged amino acid. It is possible that the divalent metal cofactor normally coordinates with the phosphate group of the sugar to allow efficient binding to the ε-amino group of the lysine (and to a lesser extent with an arginine residue). However, the negative charge of glutamate could also be neutralized by metal coordination via water groups. Perhaps, unlike ADP-glucose pyrophosphorylase, the phosphate pocket of PMI-GMP supports metal coordination with glutamate. Replacing lysine by metal coordination  via water groups. Perhaps, unlike ADP-
require an allosteric activator for function. Since Haugen and Preiss (47) found that ADP-glucose pyrophosphorylase first binds ATP and then binds β-glucose 1-phosphate, it follows that β-mannose 1-phosphate binding may also be reduced for the Arg-19 mutants if the binding of GTP is required prior to binding of the sugar phosphate.

The ADP-glucose pyrophosphorylase allosteric site is highly basic, and at least 1 other lysine residue and 1 arginine residue have been implicated in the allosteric regulation of this enzyme (39, 48, 49). It is thought that the cationic groups together effectively reduce the pK of the ε-amino group of Lys-39 and constitute the anion binding site (48). For PMI-GMP, Arg-19 is an important residue for GMP activity, and it is possible that several neighboring residues (e.g. Arg-13, Lys-20, and Lys-24) contribute to the basic nature of this region. Guanidinium groups have been shown to bind phosphate (50), and there is a precedent for the involvement of arginyI residues in the binding sites of anionic substrates and cofactors (51-53). Thus, Arg-19 and the surrounding region have the characteristics of an anion binding site that, perhaps, is involved in the binding of GTP and or an allosteric effector.

Plasmid pSA12 required IPTG induction to restore strain 8853 containing algA mutant plasmids (Table I) grown in the presence or absence of IPTG was either mucoid, alginate-producing (M), or nonmucoid (N).

b PMI-GMP purification was described under “Experimental Procedures.” PMI activity was measured in the reverse direction, and GMP activity was measured in the forward direction relative to alginate synthesis (16).

The K175Q mutant no longer shows the preference for colbalt over magnesium for the PMI activity which is observed for the wild type PMI-GMP and the rest of the mutant proteins.

**Table II**

| PMI-GMP protein | Phenotype* | V_{max} | Apparent K_{m} | GTP |
|-----------------|------------|---------|----------------|-----|
| *IPGT  | -IPGT  | PMI  | GMP  | M6P  | MIP  |
| Wild type  | M  | M  | 3,711 | 3,423 | 2.9  | 8.2  | 41.2 |
| K175R  | M  | N  | 5,066 | 3,376 | 2.1  | 3,830.00 | 75.4 |
| K175Q  | M  | N  | 5,815 | 1,360 | 6.3  | 26,200.00 | 122.9 |
| K00Q  | N  | N  | 5,777 | 291  | 1.4  | 5,010.00 | 78.1 |
| K19H  | N  | N  | 2,963 | 3,423 | 5.4  | 13.3  | 44.8 |
| K19R  | N  | N  | 3,465 | 1,386 | 2.3  | 66.3  | 287.0 |
| K19L  | N  | N  | 3,460 | 1,497 | 2.0  | 21.1  | 238.3 |
| S12A  | M  | N  | 4,385 | 1,907 | 1.8  | 41.1  | 189.4 |
| 321  | 565  | 1,525 | 0.8  | 1.7  | 12.3  |

* The phenotype of 8,853 containing algA mutant plasmids (Table I) grown in the presence or absence of IPTG was either mucoid, alginate-producing (M), or nonmucoid (N).

**In Summary**—The major goal of this study was to define the regions of PMI-GMP responsible for each of the enzymatic activities. It is apparent that the amino-terminal half of the protein contains at least two regions critical for GMP enzyme activity. Lys-175 is involved in the binding of β-mannose 1-phosphate, presumably via interaction of the phosphate group with the ε-amino group of Lys-175, and Arg-19 probably forms part of the GTP binding site. The homology of the amino-terminal half of PMI-GMP with several pyrophosphorylases adds further support for the idea that the amino-terminal portion is important for GMP activity. The chymotrypsin studies indicate that the carboxyl-terminal region of the xanB protein is important for both PMI and GMP activities. The results also show that some parts of the protein (i.e. Ser-12 and Val-321) are important for both PMI and GMP activities. Identifi-
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