Purification and Characterization of Guanosine Diphospho-D-mannose Dehydrogenase

A KEY ENZYME IN THE BIOSYNTHESIS OF ALGINATE BY PSEUDOMONAS AERUGINOSA

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Siddhartha Roychoudhury†, Thomas B. May†, John F. Gill‡, Shrikrishna K. Singh§, David S. Feingold*, and A. M. Chakrabarty††

From the †Department of Microbiology and Immunology, University of Illinois College of Medicine, Chicago, Illinois 60612 and the ‡Department of Microbiology, Biochemistry, and Molecular Biology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261

Alginic acid-producing Pseudomonas aeruginosa are usually associated with the cystic fibrosis lung environment and contribute to the high mortality rates observed among these patients. The present paper describes the purification and enzymatic properties of guanosine diphospho-D-mannose dehydrogenase (EC 1.1.1.132), a key enzyme in alginate biosynthesis by mucoid P. aeruginosa. The enzyme was overproduced using a plasmid vector containing algD (the gene encoding this enzyme) under control of the tac promoter. It was purified from cell-free lysates by lowering the pH to 5.0, heating the extract to 57.5 °C for 10 min, and discarding the protein pellet. The enzyme was selectively precipitated from the supernatant fraction with 45% acetone, resuspended in a 100 mM triethanolamine acetate buffer, pH 7.6, and ultimately purified using Bio-Sil TSK-400 gel filtration chromatography. The subunit molecular weight (M, 48,000) as well as the N-terminal amino acid sequence corresponded to those predicted from the DNA sequence of algD. The native protein migrated as a hexamer of 290,000 molecular weight upon Bio-Gel A-1.5m gel filtration chromatography. Kinetic analysis demonstrated an apparent K_m for the substrate GDP-D-mannose and 185 μM for the cofactor NAD*. GDP-D-mannuronic acid was identified as the enzyme reaction product. Several compounds (including GMP, ATP, GDP-D-glucose, and maltose) were found to inhibit enzymatic activity. GMP, the most potent of these inhibitors, exhibited competitive inhibition with an apparent K_i of 22.7 μM. Enzyme activity was also sensitive to the sulfhydryl group modifying agents iodoacetamide and p-hydroxymercuribenzoate. The addition of excess di-thiothreitol restored enzyme activity, suggesting a possible involvement of cysteine residues in enzymatic activity.

Chronic pulmonary infection by mucoid alginate-producing Pseudomonas aeruginosa is a leading cause of mortality among patients suffering from cystic fibrosis (CF) (1). Alginate-producing P. aeruginosa are primarily associated with the CF lung environment and are particularly deleterious to CF patients for several reasons. First, alginate interferes with the host defense mechanisms by inhibiting phagocytosis of P. aeruginosa (2-6), preventing antibody coating (7), and impeding chemotaxis of polymorphonuclear leukocytes (8). Second, alginate provides a barrier against penetration of aminoglycoside antibiotics (9, 10). Third, alginate complicates the bronchial obstruction and contributes to increased deterioration of the CF lung (11-13). Inhibition of alginate biosynthetic enzymes with nontoxic agents may prove useful in eliminating the protective alginate barrier, thereby allowing successful antibiotic treatment of P. aeruginosa infections in the lungs of CF patients.

Alginate is a linear copolymer consisting of β-1,4-linked D-mannuronic acid and variable amounts of the C-5 epimer L-guluronic acid (14). Piggott et al. (15) and Padgett and Phibbs (16) initially demonstrated the presence, albeit at low levels, of key enzymes involved in alginate biosynthesis by P. aeruginosa. However, attempts to purify and characterize these enzymes have generally failed due to the extremely low enzyme activities observed in even the most heavily mucoid alginate-producing strains of P. aeruginosa. We have extended the studies of the alginate pathway (see Fig. 1) by using a molecular genetic approach to increase enzyme yields (17-23) as well as to investigate regulatory mechanisms controlling their synthesis (21, 24-26). GMD, which is encoded by the algD gene, is of particular importance in alginate biosynthesis (21). The product of an alginate regulatory gene (algR) has been found to activate the algD promoter (26). Indeed, transcriptional activation of algD is a prerequisite for alginate production by P. aeruginosa (21, 26).

Although GMD has been partially purified from Arthrobacter (27), it has not been isolated from mucoid strains of P. aeruginosa. Deretic et al. (21) previously constructed the plasmid pVD211 by cloning the algD gene directly downstream of the tac promoter in the broad host range controlled expression vector pMBB24. IPTG induction of cells harboring pVD211 resulted in the hyperproduction of a 48,000 molecular weight polypeptide (as predicted from DNA sequence analysis (26)) which correlated with an increase in GMD activity (21). The

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† Postdoctoral Fellow of the Cystic Fibrosis Foundation. Present address: Boehringer Mannheim, P.O. Box 50816, Indianapolis, IN 46250.

‡ To whom correspondence should be sent: Dept. of Microbiology and Immunology, University of Illinois College of Medicine, 835 S. Wolcott Ave., Chicago, IL 60612.

1 The abbreviations used are: CF, cystic fibrosis; GMD, GDP-D-mannose dehydrogenase; IPTG, isopropyl β-D-thiogalactopyranoside; HPLC, high performance liquid chromatography.
cloning and overexpression of GMD have enabled us to purify and characterize this key alginate biosynthetic enzyme.

**EXPERIMENTAL PROCEDURES**

**Materials**

Sodium dodecyl sulfate, acrylamide, bisacrylamide, Bio-Sil TSK-400 HPLC gel filtration column, Bio-Gel A-1.5m gel filtration medium, anion exchange AG 1-×8 resin, Coomassie Blue R-250, and Bradford protein assay reagent were from Bio-Rad. All chemicals (including IPTG, GDP-D-mannose, NAD*, d-mannuronic acid, ido- acetamide, and p-hydroxymercuribenzoate) were obtained from Sigma unless stated otherwise. L-Lactate dehydrogenase (EC 1.1.1.27), alkaline phosphatase (EC 3.1.3.1), and phosphodiesterase (EC 3.1.4.1) were also purchased from Sigma. Centricon-10 microcentrators were from the Amicon Division of W. R. Grace & Company (Danvers, MA). A native molecular weight marker kit was obtained from United States Biochemical Corp. (Cleveland, OH). Cellulose and phosphoethylemineine thin layer chromatography plates with fluorescent indicator were from Brinkmann Instruments and Machinery-Nagel (Duren, Federal Republic of Germany), respectively. GDP-D-[3,4-3H]mannose, GDP-D-[U-14C]mannose, and Aquasol scintillation mixture were from Du Pont-New England Nuclear.

**Strains and Plasmids**

P. aeruginosa 8821 is an alginate-producing (Alg*) strain isolated from the spumotum of a CF patient (17). Strain 8822 is a spontaneous non-mucoid (Alg-) derivative of strain 8821. Darzans and Chakraborty (17) previously isolated a stable Alg* strain 8830 via chemical mutagenesis of the spontaneous non-mucoid strain 8821. Strain 8835 is an Alg*, GMD* mutant obtained by further mutagenesis of strain 8830 (17). Deretic et al. (22) have described the construction of the plasmid pVD211 which carries the algD gene (encoding GMD) on a 3.2-kilobase ClaI-BglII fragment that had been converted into a BamHI site (see Fig. 1). Strain 8835 carrying the plasmid pVD211 demonstrates an Alg*, GMD* phenotype upon induction with IPTG (21).

**GMD Assay**

GMD activity was assayed by monitoring the NAD* reduction rate at 340 nm at 24 °C in a Gilson model 2600 spectrophotometer (28). The assay reaction mixture contained 50 mM Tris-Cl buffer, pH 8.0, 0.9% NaCl, and 100 mM sodium phosphate, pH 7.0, NAD* (1 mM), GDP-D-mannose (10−20 μl (25−50 μg of protein)) of enzyme) in a total volume of 1.0 ml. A protein assay contained the identical reaction mixture except that the substrate GDP-D-mannose was omitted. The rate of NAD* reduction in the absence of GDP-D-mannose was subtracted from the rate observed in the presence of substrate to correct for endogenous dehydrogenase activity. The level of endogenous activity was 0.4 and 0.0 milliunits/ml for the crude extract (step 1 below) and purified enzyme, respectively. One unit of enzyme activity was defined as 1 μmol of NAD* reduced per min under the assay conditions. Specific activity of the enzyme is reported in milliunits per mg of protein. Protein concentration was determined by the method of Bradford with bovine serum albumin as a standard (29).

**Purification of GDP-D-mannose Dehydrogenase**

The following protocol was used to purify GMD. All steps were performed at 4 °C unless otherwise indicated.

**Step 1: Preparation of Crude Extract—P. aeruginosa 8835 containing the algD expression vector pVD211 was used as the source of GMD. Cells grown in M-9 media (per liter) with vigorous shaking (250 rpm). The growth media contained 300 mg/liter of carbenicillin to maintain a selective pressure for pVD211. IPTG (1 mM final concentration) was added to the cultures after an initial 2-h growth period. The incubation was then continued for an additional 3 h, at which time an algD transcription from the tac promoter. The cells were then harvested by centrifugation at 8,000 × g for 10 min. The cell pellet (approximately 5 g, wet weight) was washed once with 0.9% NaCl and resuspended in 4.5 ml of lysis buffer (50 mM Tris acetate, pH 7.6, 1 mM phenylmethylsulfonyl fluoride, 5 mM dithiothreitol). The cells were disrupted by three passages through a French pressure cell at 14,000−16,000 p.s.i. and then centrifuged at 40,000 × g for 30 min. The resulting supernatant was recentrifuged at 105,000 × g for 10 min. This clarified supernatant was treated with protamine sulfate (1% final concentration) and immediately centrifuged at 20,000 × g for 10 min to remove DNA. The final supernatant (approximately 4 ml) is referred to as crude extract.

**Step 2: pH and Heat Treatment—The crude extract from step 1 was diluted with 0.5 volume of 1 M Tris acetate, pH 5.0, mixed well, and then incubated at 55 °C for 10 min. The precipitate was removed by centrifuging the mixture at 20,000 × g for 10 min at 4 °C.

**Step 3: Acetone Precipitation—The supernatant from step 2 (5.0 ml) was then treated with 0.45 volume of acetone, incubated on ice for 3 min, and recentrifuged. The supernatant was discarded. The precipitate was resuspended in 5.5 ml of 100 mM triethanolamine acetate buffer, pH 7.6, 1 mM phenylmethylsulfonyl fluoride, and 2 mM diethiothreitol. A 1.0-ml aliquot (6 mg of protein) of the enzyme solution from step 3 was applied to the column. Protein was eluted from the column at a flow rate of 0.3 ml/min. Fractions of 0.9 ml were collected and monitored for GMD activity and protein content. The process was repeated four times for the remaining step 3 enzyme sample. The single peak fractions from each column run were pooled together.

The purity and subunit molecular weight of GMD were ascertained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to the method of Laemmli (30) using a 13% acrylamide resolving gel and a 5% acrylamide stacking gel. Gels were stained for protein by Coomassie Blue R-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 was estimated by gel filtration chromatography on a Bio-Gel A-1.5m column (24 × 3 cm). The column was equilibrated with a 100 mM potassium phosphate buffer, pH 7.0, containing 200 mM NaCl. The column was then calibrated with 50 μl of a native molecular weight marker kit containing 10 μg each of lysozyme (140,000), L-lactate dehydrogenase (M, 290,000), enolase (M, 67,000), adenylate kinase (M, 32,000), and cytochrome c (M, 12,400). The column flow rate was maintained at 0.5 ml/min. Each 2.0-ml fraction was assayed for protein content. A 16-μl (20 μg of protein) aliquot of the GMD enzyme preparation (step 4) was then applied to the column. GMD activity and protein content were measured for each fraction.

**Identification of Reaction Products**

GMD reaction mixtures were similar to those described above except that NAD* was supplied in 2.5-fold excess. Each reaction mixture contained 150 μl of either GDP-D-mannose or step 1 purified enzyme and 0.5 μCi/ml of either GDP-D-[3,4-3H]mannose (29.1 Ci/mmol) or GDP-D-[U-14C]mannose (0.3 Ci/mmol). Aliquots of the radiolabeled substrate were dried as directed by the supplier and resuspended in an equivalent volume of water prior to use. Reaction mixtures were incubated at 25 °C until no further increase in uronic acid content was observed (2 h). Uronic acid content was determined according to the method of Knutson and Jeanes (31) using D-mannuronic acid as the standard. Some samples were then treated with 50 units of L-lactate dehydrogenase and 50 μmol of sodium pyruvate to remove NADH and its acid degradation products (27). Samples were deproteinized with Centricon-10 microcentrators and stored at −70 °C until use. Control reaction mixtures were also prepared with boiled enzyme for comparative purposes.

GMD reaction mixtures (scaled up to 5 ml) were applied to a Dowex 1 × 8 column (formate form, 200−400 mesh, 15 × 0.9 cm). The reaction constituents were eluted (0.5 ml/min, 2.0-ml fractions) with a 400-ml linear gradient of 0–1.6 M ammonium formate, pH 5.0 (32). Nucleotide-containing compounds were detected by absorbance at 260 nm. Radioactivity was determined by dissolving 0.1 ml of sample in 5 ml of Aquasol scintillation cocktail and counting for 3H or 14C. Ammonium formate concentration was measured by conductivity. Samples were desalted (27) by adsorption on Norit A charcoal, washing with water, eluting with 50% ethanol, 1% NH4OH, and concentrating approximately 20-fold with a Savant SpeedVac concentrator prior to determination of uronic acid, phosphate, and guanosine content. Total and acid-labile phosphate were measured and compared with GDP-D-mannose standards (33, 34). Guanosine content
was determined by its characteristic absorption spectrum at pH 1, 7, and 12 (27, 32, 35).

Sugar nucleotides were also separated from GMD reaction mixtures by paper electrophoresis on EDTA-washed Whatman No. 1 filter paper in 0.2 M ammonium acetate, pH 6.0, and by thin layer (0.1-mm) chromatography on cellulose (solvent A, 95% ethanol, 1 M ammonium acetate, pH 7.5 (3:2:3)). Uronic acids were separated by paper electrophoresis or by paper chromatography (Whatman No. 1) (solvent B, acetonitrile, isopropanol, 95% ethanol, water (3:1:1:1); solvent C, ethyl acetate:pyridine:water (5:3:1:1); and solvent D, 1-propanol:ethyl acetate:water (7:2:2)). Sugar nucleotides were identified on thin layer cellulose plates (solvent A) or on two-dimensional phosphoethylamine plates (solvent E, 0.4 M guanidinium hydrochloride; and solvent F, 5.6 M ammonium sulfate, 35 mM ammonium bisulfate, 107 mM sodium EDTA) (36). Hexoses were separated by paper chromatography in solvent D. Nucleotides and nucleosides were detected at 254 nm. Carbohydrates were detected with alkaline silver nitrate spray (37). Labeled compounds were magnified with a 4x strip counter (Tracerlab, Waltham, MA) or by autoradiography on Kodak (no screen) XAR-5 film.

RESULTS

GMD Purification and Characterization—The purification of GMD was facilitated by the ability to overexpress the algD gene from a previously constructed expression vector pVD211 (Fig. 1) (21). Table I shows that IPTG induction of 8835/pVD211 resulted in a 12-fold enrichment in GMD activity over that observed for the original Alg' CF isolate strain 8821. GMD activity was 200-fold higher than observed in uninduced cultures of 8835/pVD211. The purification of GMD was obtained in four steps (Table II, Fig. 2, and Fig. 3): preparation of the crude extract; pH/heat treatment; acetone precipitation; and gel filtration. The considerable heat stability of GMD at an acidic pH (5.0) was a property that expedited removal of the bulk of contaminating proteins (Fig. 3). These purification steps resulted in an 11-fold increase in GMD specific activity. Thus, the total purification was 134-fold over the initial activity detected in 8821 and 2100-fold over that in uninduced 8835/pVD211. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the purified protein showed a single polypeptide with a molecular weight of 48,000 (Fig. 3). The native protein was estimated to be a hexamer of 290,000 molecular weight. The subunit molecular weight agreed with values previously obtained by maxicell and DNA sequence analysis (21, 26). In addition, the N-terminal amino acid sequence determined from this purified protein directly matched the protein predicted from the algD DNA sequence (Fig. 4).

Kinetic parameters were evaluated from double-reciprocal plots (Fig. 5), and under the reported assay conditions, the apparent Km and Vmax values were 14.9 μM and 581.4 nmol of GDP-d-mannose oxidized per min per mg of enzyme, respectively. The apparent Km for NAD+ was 185 μM. NADP+ did not serve as a cofactor for GMD activity. In addition, GMD appeared to be quite specific for GDP-d-mannose since it did not utilize d-mannose, UDP-d-mannose, UDP-d-glucose, TDP-d-glucose, GDP-d-glucose, GDP-d-glucose, UDP-d-xylose, ethanol, 6-glyceraldehyde 3-phosphate, L-lactate, or l-histidinol as alternative substrates. The pH and temperature optima for GMD activity were 7.7 and 50 °C, respectively. The pH profile yielded an unusually sharp peak and indicated that GMD activity is highly sensitive to pH changes. Although GMD activity was remarkably heat stable, activity was abolished within 2–3 min of raising the temperature above the optimum.

A number of nucleoside phosphates and sugars were tested as inhibitors of GMD activity (Table III). GMP was the most potent inhibitor identified. GMP was a competitive inhibitor with an estimated Ki of 22.7 μM (Fig. 5). GMD was also treated with a 10-fold excess of either p-hydroxymercuribenzoate or iodoacetamide, preincubated on ice for various time periods and then assayed for enzyme activity. Treatment with p-hydroxymercuribenzoate resulted in 96% inactivation after a 5-min preincubation. Iodoacetamide showed a similar but a less pronounced effect on GMD activity. Inactivation was 40% after 30 min of preincubation. In both cases, GMD was fully reactivated by treating the mixture with excess (25 mM) dithiothreitol. These results indicated a possible involvement of sulphydryl groups (cysteine residues) in enzymatic activity.

Characterization of GDP-d-mannose Oxidation Products—GMD reaction mixtures were separated by Dowex 1-X8 column chromatography (Fig. 6). Peak 8 was observed only in the complete reaction mixtures and was tentatively identified as GDP-d-mannuronic acid based on the presence of 3H label and a positive uronic acid test. L-Lactate dehydrogenase/pyruvate treatment removed the NADH shoulder, allowing better separation of peak 8. No differences were observed using step 2 or purified enzyme sources. The UV absorption spectra at pH 1, 7, and 12 suggested the presence of guanosine, and chemical analysis indicated a molar ratio of 2 total phosphates, 1 acid-labile phosphate, 1 guanosine, and 1 uronic acid (Table IV).
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**TABLE II**

| Purification step | Volume | Protein concentration | Specific activity | Purification factor | Yield |
|-------------------|--------|-----------------------|------------------|---------------------|-------|
| 1. Crude extract  | 4.0    | 7.6                   | 523              | 65                  | 80    |
| 3. Acetone precipitation | 5.5 | 5.4                   | 592              | 74                  | 72    |
| 4. Bio-Sil TSK-400 column | 0.9 (× 5) | 1.5                   | 1068             | 134                 | 29    |

**DISCUSSION**

This paper describes the purification and enzymatic properties of GDP-D-mannose dehydrogenase from alginate-producing strains of *P. aeruginosa*. GMD was found to exhibit a number of characteristics common to other four-electron transfer dehydrogenases that (i) oxidize a primary alcohol group to a carboxyl group with NAD(P)+ as a cofactor; (ii) consist of an even number of identical subunits of approximately 50,000 molecular weight; and (iii) utilize sulfhydryl groups for catalytic activity (39).

GMD was found to catalyze the NAD+ dependent oxidation of GDP-D-mannose to form GDP-D-mannuronic acid. These results suggest that GMD plays a crucial role in synthesis of the glycosyl donor for subsequent polymerization events (27). The enzyme appeared to be specific for GDP-D-mannose and the NAD+ cofactor since dehydrogenase activity was not observed with several other sugars, nucleotide sugars, or NADP+. In addition, GMP was shown to inhibit GMD activity competitively, whereas mannose acted neither as an inhibitor nor an alternative substrate of GMD. This suggested that the guanosine moiety binds the enzyme and perhaps induces a conformational change necessary to make the active site accessible to the mannose moiety. The inhibition affected by GDP-D-glucose is consistent with this notion. However, GDP and GTP did not significantly inhibit enzyme activity, suggesting that the additional negative charge of GDP and GTP may prevent their entry into the active site. Interestingly, the first step of polymerization reactions often reversibly releases...
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**FIG. 4.** Amino acid sequence of GMD as predicted from the algD DNA sequence (26). N-terminal sequence is boxed. Regions homologous to UDP-D-glucose dehydrogenase from bovine liver (W) and to L-histidinol dehydrogenase from *E. coli* (n) (39) are shown. Dashed lines indicate other amino acids in the catalytic sites of these dehydrogenases. SC (inside circle) is also present in the catalytic site of *α*-glyceraldehyde-3-phosphate dehydrogenase (39). Cys-268 is indicated by the arrow. Regions homologous to the conserved sequences (*) of ATP-binding proteins are shown (42). The actual ATP-binding consensus sequence is underlined.

**FIG. 5.** Kinetics of GMP inhibition of GMD. ○, ■, and ▲ refer to 0, 50, and 100 μM GMP, respectively. An 8-μg aliquot of purified GMD was used in each assay. Standard assay conditions are described under "Experimental Procedures." One milliunit/mg refers to 0.5 nmol of GDP-D-mannose oxidized per min per mg of protein.

**TABLE III**

Inhibitors of GMD activity

| Compound | Substrate concentration | Inhibitor concentration | Inhibition |
|----------|------------------------|-------------------------|-----------|
|          | mM                     | mM                     | %         |
| GMP      | 1                      | 0.1                     | 9.9       |
|          | 1                      | 1.0                     | 51.9      |
|          | 1                      | 5.0                     | 92.0      |
| ATP      | 1                      | 0.1                     | 10.0      |
|          | 1                      | 1.0                     | 25.5      |
|          | 1                      | 5.0                     | 62.7      |
| GDP-D-glucose | 1            | 0.1                     | 7.5       |
|          | 1                      | 1.0                     | 25.1      |
|          | 1                      | 5.0                     | 61.2      |
| Maltose  | 1                      | 5.0                     | <0.5      |
|          | 1                      | 10.0                    | 17.4      |

**FIG. 6.** Dowex 1-X8 column profile of GMD reaction mixture components. The samples loaded onto the column were 5-ml reaction mixtures using step 2-purified enzyme as described under "Experimental Procedures." Samples were: complete reaction mixture (top panel), boiled enzyme reaction mixture (middle panel), and L-lactate dehydrogenase/pyruvate-treated complete reaction mixture (bottom panel). $A_{280\text{~nm}}$ (---), $^3\text{H}$ radioactivity (---), ammonium formate concentration (- - -), uronic acid content rated on a relative scale of 0-10 (inset). Peaks 2, 5, and 7 migrated with authentic NAD*, GDP-D-mannose, and NADH, respectively.

GMP (40), suggesting a possible feedback control mechanism of GMD activity.

GMD appears to consist of six identical subunits ($M_0$, 48,000). As shown in Fig. 4, the amino acid sequence around Cys-268 of GMD shows homology to the catalytic sites of two other NAD*-linked, four-electron transfer dehydrogenases: UDP-D-glucose dehydrogenase from bovine liver and L-histidinol dehydrogenase from *Escherichia coli* (39, 41). The reaction mechanism proposed for UDP-D-glucose dehydrogenase involves a two-step process in which the enzyme sequentially acts as an alcohol dehydrogenase and as an aldehyde dehydrogenase. GMD may share a similar evolutionary hi-
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An analysis of the material in peak 8 from the Dowex 1-X8 column.

Peak 8 was from the Dowex 1-X8 column profile shown in Fig. 6. The peak fractions were pooled, desalted, concentrated, and assayed according to the protocols described under “Experimental Procedures.” Theoretical values for guanosine are λmax = 252-253 nm, 250:260 = 1.12, 290:260 = 0.65, and 290:290 = 0.28 at pH 7.0 (27, 32, 35).

| Assay      | Amount (μmol/ml) | Molar ratio | pH | λmax (nm) | 250:260 | 280:260 | 290:290 |
|------------|------------------|-------------|----|-----------|---------|---------|---------|
| Total PO4  | 0.653            | 2.00        | 1  | 256       | 1.02    | 0.67    | 0.50    |
| Acid-labile| 0.330            | 0.92        | 7  | 253       | 1.14    | 0.61    | 0.27    |
| Guanosine  | 0.365            | 1.12        | 12 | 259       | 0.90    | 0.62    | 0.23    |
| Uronic acid| 0.332            | 1.02        |    |           |         |         |         |

* The ratio of absorbance at various wavelengths (nm).

The reversible inactivation of GMD by sulfhydryl modifying agents p-hydroxymercuribenzoate and iodoacetamide suggested a role for cysteine residues in enzymatic activity, possibly in the active site. Sulfhydryl groups of cysteine are often involved in the catalytic activity of four-electron transfer dehydrogenases (39). As mentioned above, the region containing Cys-268 (Fig. 4) showed homologies to the catalytic sites of other four-electron transfer dehydrogenases and thus may be involved in GMD catalytic activity. However, these inhibitors may also affect enzyme activity at other cysteine residues by altering interactions critical to protein conformation.

The results of this study have raised a number of interesting questions regarding the nature of the active site of GMD. Site-directed mutagenesis experiments are presently under way to determine if Cys-268 is in fact involved in substrate binding and/or GMD catalytic activity. Similar experiments may determine the binding site for the NAD+ cofactor. The availability of GDP-d-mannuronic acid (via GMD activity toward GDP-d-mannose) has now allowed us to study the incorporation of this precursor into alginate polymer. These studies should further our understanding of GMD and the subsequent polymerization reactions of alginate biosynthesis by mucoid strains of P. aeruginosa.

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