Pseudomonas aeruginosa C5-Mannuronan Epimerase: Steady-State Kinetics and Characterization of the Product†

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ABSTRACT: Alginate is a major constituent of mature biofilms produced by *Pseudomonas aeruginosa*. The penultimate step in the biosynthesis of alginate is the conversion of some β-D-mannuronate residues in the polymeric substrate polymannuronan to α-L-guluronate residues in a reaction catalyzed by C5-mannuronan epimerase. Specificity studies conducted with size-fractionated oligomannuronates revealed that the minimal substrate contained nine monosaccharide residues. The maximum velocity of the reaction increased from 0.0018 to 0.0218 s^{-1} as the substrate size increased from 10 to 20 residues, and no additional increase in k_{cat} was observed for substrates up to 100 residues in length. The K_m decreased from 80 μM for a substrate containing fewer than 15 residues to 4 μM for a substrate containing more than 100 residues. In contrast to C5-mannuronan epimerases that have been characterized in other bacterial species, *P. aeruginosa* C5-mannuronan epimerase does not require Ca^{2+} for activity, and the Ca^{2+}−alginate complex is not a substrate for the enzyme. Analysis of the purified, active enzyme by inductively coupled plasma-emission spectroscopy revealed that no metals were present in the protein. The pH dependence of the kinetic parameters revealed that three residues on the enzyme which all have a pK_a of ∼7.6 must be protonated for catalysis to occur. The composition of the polymeric product of the epimerase reaction was analyzed by ^{1}H NMR spectroscopy, which revealed that tracts of adjacent guluronate residues were readily formed. The reaction reached an apparent equilibrium when the guluronate composition of the polymer was 75%.

Pseudomonas aeruginosa is an opportunistic human pathogen that has been linked to certain pathological conditions of immunocompromised humans, and especially the respiratory tract infections that accompany cystic fibrosis (*1*). Pulmonary infections caused by the mucoid phenotype of *P. aeruginosa* are almost impossible to eradicate, even with antibiotic treatment (*2*). The strong antibiotic resistance is attributed in part to the formation of a biofilm, a complex extracellular polymeric matrix in which the cells are embedded. One of the primary constituents of the mature *P. aeruginosa* biofilm is the polysaccharide alginate. This cell-associated virulence factor is a high-molecular mass (500−2000 kDa) linear polysaccharide comprised of residues of β-D-mannuronate (M)[1] and its C-5 epimer α-L-guluronate (G), which are covalently linked by β-1,4-glycosidic bonds (*3*). The relative ratio of these building blocks in the polymer and the linear distribution of G residues strikingly alter physical properties of alginate such as viscosity and gel forming ability, and therefore play a crucial role in the function of the biopolymer (*4*).

Most of the genes required for alginate biosynthesis are located in the *algD* operon on the *P. aeruginosa* chromosome (*5*). The first polymeric product in the pathway is polymannuronan, which is synthesized from GDP-mannuronic acid. The formation of GDP-mannuronic acid from fructose 6-phosphate occurs in the cytoplasm; the synthesis of polymannuronan is not well understood, but two cytoplasmic membrane proteins, Alg8, which is a β-glycosyltransferase-like protein, and Alg44, are believed to be involved in polymer formation (*6*). Polymannuronan is transported across the inner cytoplasmic membrane, and conversion of some mannuronate residues in the polymer to guluronate occurs in the periplasmic space. The reaction is catalyzed by C5-mannuronan epimerase (Scheme 1), which is a 55 kDa protein encoded by the *algG* gene (*7*). Mature alginate is acetylated at some mannuronate residues at O2 or O3 (*8*). The acetylated residues are not substrates for the epimerase, and guluronate residues are not acetylated; therefore, the final composition of the alginate polymer is determined by the relative activities of the epimerase and the acetyltransferases that act on alginate. Following acetylation, the polysaccharide is exported across the outer membrane by the porin-like protein AlgE (*9*).

Mature alginate that is isolated from *P. aeruginosa* biofilms has G and M residues randomly distributed throughout the polymer, and homopolymeric G blocks or M blocks are absent (*10*). On the basis of the composition of *P. aeruginosa* alginate, it is widely believed that C5 mannuronan epimerase cannot catalyze the epimerization of adja-

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[1] Abbreviations: dp, degree of polymerization or number of monosaccharide units in a polysaccharide or oligosaccharide; G, guluronate; M, mannuronate; poly-M, polymer of mannuronate residues; poly-G, polymer of guluronate residues; IPTG, isopropyl β-D-thiogalactopyranoside; PMSF, phenylmethanesulfonyl fluoride; TLCK, *N*-tosyl-L-lysine chloromethyl ketone; DTT, dithiothreitol.

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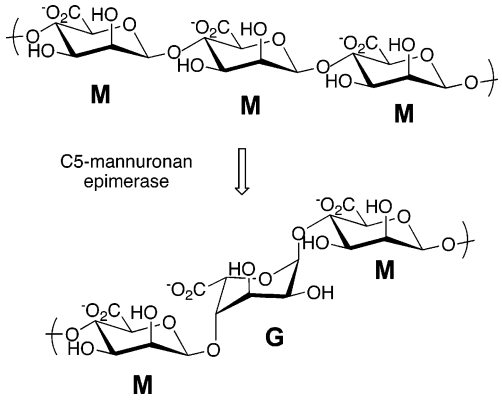
Scheme 1



cent residues to form poly-G blocks. In contrast, the epimerases from *Azotobacter vinelandii* have been demonstrated to form poly-G blocks (*11*, *12*). *A. vinelandii* contains several C5-mannuronan epimerases, which are Ca^{2+}-dependent, and highly homologous with one another (*13*). *P. aeruginosa* mannuronan epimerase is not closely related to the *A. vinelandii* enzymes, and although its activity has been reported to be Ca^{2+}-dependent, that aspect of the reaction has not been examined in detail. Sequence analysis and homology modeling of the *P. aeruginosa* epimerase suggest that the epimerase domain of the protein is a right-handed β-helix, which is characteristic of enzymes that utilize polysaccharides as substrates (*14*). A variety of kinetic studies are presented here, which provide insight into the interactions between the epimerase and alginate. The substrate size specificity and metal ion dependence of the reaction have been defined. Through ^{1}H NMR analysis of the product, we have investigated the sequential distribution of G residues with respect to the fractional content of the polymeric chain. Our results suggest that the enzyme does not require Ca^{2+} for activity, and that it is capable of forming alginate containing poly-G blocks.

EXPERIMENTAL PROCEDURES

Cloning, Overexpression, and Purification of C5-Mannuronan Epimerase. The *algG* gene was successfully amplified from *P. aeruginosa* PAO1 genomic DNA and inserted into the pET-14b expression vector. The PCR protocol used for amplification of DNA from the GC-rich genome of *P. aeruginosa* has been described previously (*15*). *Escherichia coli* strain JM109 was used for cloning and maintaining the plasmid. For protein production, the recombinant plasmid was transformed into *E. coli* BL21(DE3)pLysS cells. Cells were grown in LB medium supplemented with ampicillin (100 μg/mL) and chloramphenicol (34 μg/mL) at 30 °C with rotary shaking until the OD_{600} reached 0.8. Subsequently, 400 μM IPTG was added to induce expression of C5-mannuronan epimerase, and growth of the cells was continued at 30 °C for 16 h. Cells were harvested by centrifugation (6500*g* for 15 min), yielding 25 g of cell paste, and resuspended in 130 mL of lysis buffer containing 100 mM MOPS (pH 7.5), 100 mM NaCl, 1 mM β-mercaptoethanol, and 10 mM imidazole. Bacterial lysis was achieved by repeated freeze−thaw cycles of the resuspended cells in the presence of 0.5 mM PMSF and 0.5 mM TLCK to inhibit protease activity. To lower the viscosity of the cell-free extract, 1.3 mg of DNAse was added, along with 2 mM $CaCl_2$ and 2 mM $MgCl_2$. After the insoluble cell debris was removed by centrifugation (12200*g* for 60 min), the cell-free extract was loaded onto a Ni−NTA affinity column (30 mL, Qiagen). The resin-bound His_6-tagged fusion protein was washed with 30 column volumes of buffer containing 100 mM MOPS (pH 7.5), 300 mM NaCl, 1 mM β-mercaptoethanol, and 10 mM imidazole, followed by 30 column volumes of buffer containing 100 mM MOPS (pH 7.5), 300 mM NaCl, 1 mM β-mercaptoethanol, and 50 mM imidazole. C5-Mannuronan epimerase was eluted from the column with a buffer containing 100 mM MOPS (pH 7.5), 300 mM NaCl, 1 mM β-mercaptoethanol, and 500 mM imidazole. The His_6 affinity tag was cleaved from the fusion protein by thrombin digestion, which was conducted at 4 °C using 1 unit of protease for every 5 mg of epimerase at an epimerase concentration of 3 mg/mL. Under these conditions, the reaction was complete within 48 h. Subsequently, thrombin was removed by passing the solution over a *p*-aminobenzamidine−agarose column (Sigma Chemical Co.). The purified C5-mannuronan epimerase at a concentration of 2.5−3 mg/mL was dialyzed against the following buffers in succession: 100 mM MOPS (pH 6.8), 100 mM NaCl, 250 mM imidazole, 1 mM β-mercaptoethanol, and 1 mM EDTA (12 h); 1 L of 100 mM MOPS (pH 6.8), 100 mM NaCl, 1 mM β-mercaptoethanol, and 1 mM EDTA (12 h) and 1 L of 10 mM MOPS (pH 6.8), 100 mM NaCl, 1 mM β-mercaptoethanol, and 1 mM EDTA (12 h). The epimerase was concentrated to 5 mg/mL by ultrafiltration. Glycerol was added to the protein to a final concentration of 10% (v/v), and aliquots were stored at −80 °C.

Overexpression and Purification of L*-Guluronate Lyase.* The plasmid containing the gene for L-guluronate lyase, designated pRC5, was the generous gift of D. E. Ohman (Virginia Commonwealth University, Richmond, VA). The pRC5 plasmid contains the *aly* gene from *Klebsiella aerogenes*. For expression of guluronate lyase, *E. coli* BL21(DE3)pLysS cells were transformed with pRC5 and the cells were grown at 37 °C in 6 L of LB supplemented with ampicillin (100 μg/mL). Cells were harvested by centrifugation (6800*g* for 15 min), yielding 16.9 g of cell paste, which was resuspended in a buffer containing 100 mM MOPS (pH 7.3) and 1 mM DTT. Cell lysis was achieved by digestion with lysozyme (0.2 mg/g of cell pellet) in the presence of 0.5 mM PMSF, 0.5 mM TLCK, and DNase (10 μg/mL). Nucleic acids were removed from the cell-free extract by adding protamine sulfate to a final concentration of 0.7 mg/mL. Ammonium sulfate was added to 40% saturation, and the proteins that precipitated were discarded. The concentration of ammonium sulfate in the supernatant was increased to 80% saturation, and the suspension was centrifuged (12200*g* for 20 min). The pelleted proteins, including guluronate lyase, were resuspended in a buffer containing 100 mM MOPS (pH 7.3), 1 mM DTT, and 10% (v/v) glycerol. The enzyme was stored at −80 °C at a concentration of 20 mg/mL.

*Production and Purification of Poly-*D*-mannuronan.* The substrate for C5-mannuronan epimerase, polymannuronan, was isolated from *P. aeruginosa* strain FRD462, also the generous gift of D. E. Ohman. To maintain the *P. aeruginosa* in the mucoid form, frozen glycerol stocks were initially subcultured in media containing 1 g of sodium gluconate, 1 g of sodium glutamate, 0.15 g of Na_2HPO_4, and 0.015 g of

$MgSO_4 \cdot 7H_2O$ per 50 mL at pH 7.0. After an 18 h incubation period at 30 °C with rotary shaking, agar plates with media containing 10 mM $MgSO_4$, 16.8 mM K_2HPO_4, 7.5 mM Na_2HPO_4, and 100 mM sodium glutamate overlaid with 250 μL of 40% (v/v) glycerol per plate were inoculated with mucoid cells (100−500 μL) and incubated at 37 °C for 48 h. Colonies were scraped from the plates, and the solid material was suspended in 500 mL of 100 mM NaCl and 20 mM EDTA buffer at neutral pH. The bacterial cells were pelleted by centrifugation at 1700*g* for 5 min. The supernatant was added to an equal volume of ethanol to precipitate the exopolysaccharide. The crude product was deacetylated in a 5 mM NaOH solution at 95 °C for 0.5 h and then refluxed in 0.4 N acetic acid for 18 h. The typical yield per MAP plate was 10−20 mg of polymannuronan with a final dp ranging from 1 to 30.

Purification of Poly-D-mannuronan from Alginate. Alginic acid (25 g) (Sigma Chemical Co.) was suspended in 500 mL of 10 mM Tris (pH 8.5) containing 100 mM NaCl and 20 mg of L-guluronate lyase. The heterogeneous mixture was stirred for 48 h at room temperature and then centrifuged at 6800*g* for 30 min to produce a clear supernatant, which was mainly composed of homopolymeric M and G blocks. Poly-M and poly-G blocks were obtained by fractional precipitation as follows. HCl (1 N) was pipetted into the supernatant to lower the pH by 1 unit intervals, and after each acidifying step, any precipitated polysaccharide was pelleted by a 30 min centrifugation at 6800*g*. The white solid material (dry weight, 2.2 g) obtained by precipitation between pH 3.0 and 2.0 was 90% pure poly-M, and was further purified by refluxing the material in 1 N NaOH for 20 min. Under basic conditions, the contaminating homopolymeric guluronan was decomposed, while poly-M remained intact. Mild acid hydrolysis in 0.3 N acetic acid at 80 °C overnight, followed by ethanol precipitation, yielded 2.1 g of pure poly-D-mannuronan with a dp of 10−200.

Purification of Heteropolymeric Alginate. Alginic acid (6 g) was dissolved in 1000 mL of boiling water, and when dissolution was complete, glacial acetic acid was pipetted into the solution to a final concentration of 0.3 N. The mixture was stirred at 90 °C for 18 h and then allowed to cool. HCl (1 N) was used to slowly lower the pH, resulting in precipitation of M and G block polysaccharides, which then were removed by centrifugation. The final supernatant (pH <2.0) contained a fraction of alginate that appeared to be soluble even at very acidic pH, and dry material (1.5 g) was obtained when the aqueous solvent was removed with a rotary evaporator. ^{1}H NMR analysis of the solid revealed that this fraction was low-dp heteropolymeric alginate with repeating units of MG.

Spectrophotometric Assay for C5 Epimerization. A continuous coupled assay and a fixed-time assay were developed to monitor product formation in the epimerization reaction. Spectrophotometric assays were conducted at 25 °C in a Varian CARY 50 model single-beam spectrophotometer equipped with a thermostated cell compartment. The ternary buffer mixture used in these assays had a final composition of 20 mM Tris, 10 mM MES, and 10 mM acetic acid. Sodium chloride (100 mM) was added to reaction mixtures to increase the solubility of oligomeric and polymeric substrates. A typical assay contained 200 μM poly-D-mannuronan ($dp_{ave} = 26$) in a 1 cm path length cuvette. After thermal equilibration, 10 μL of L-guluronate lyase (2 mg/mL stock) and 20 μL of C5-mannuronan epimerase (4.9 mg/mL stock) were added to the cuvette, and the absorbance change due to glycal formation was monitored at 235 nm. The concentration of L-guluronate lyase used in the assay was optimized empirically to make epimerization the rate-determining step in the coupled assay. To determine the extent of any epimerase-independent absorbance change arising from spurious D-mannuronate lyase activity in the L-guluronate lyase, the same assay was repeated in the absence of C5-mannuronan epimerase. The rate of epimerization was then calculated as the difference between the two initial velocities. The experimentally determined maximum initial rate (absorbance per second) was divided by the molar absorptivity of the glycal product [6150 M^{-1} cm^{-1} (*16*)] and the concentration of C5-mannuronan epimerase (1.8 μM) to obtain values for the reaction velocity expressed in units of inverse seconds.

The fixed-time assay was conducted by periodically transferring 100 μL aliquots from a reaction mixture to glass tubes, and terminating the reaction by placing the tube in a sand bath heated to 170 °C. The L-guluronate content of each sample was determined by the enzymatic end-point assay using L-guluronate lyase. Comparable results were obtained with the continuous and fixed-time assays.

Chromatographic Separation and Purification of Oligo-D-mannuronans. Analytical separation of oligouronides was carried out by high-performance anion exchange liquid chromatography using a Dionex DX-500 system, equipped with a CarboPac PA-1 analytical column (4 mm × 250 mm) and a pulsed amperometric detector. Samples 25 μL in volume were injected onto the column, and the individual oligomers with a dp of 1−30 were eluted with a binary gradient of sodium acetate between 0 and 1 M at a constant sodium hydroxide concentration (100 mM).

Preparative separation of oligouronides was achieved by anion exchange chromatography using DEAE-Sephadex A-25. Poly-D-mannuronan (dp = 10−200, 700 mg) was dissolved in 1 L of a buffer containing 100 mM NaCl and 5 mM sodium phosphate (pH 7.2). The sample was loaded on the column at a flow rate of 1 mL/min and eluted with a linear salt gradient from 100 to 600 mM NaCl over 1 L at a constant sodium phosphate buffer concentration. Fractions (5.5 mL) containing poly-D-mannuronan were identified by measuring the absorbance at 210 nm. The polysaccharide was precipitated by addition of an equal volume of ethanol and pelleted by centrifugation. The pelleted material was lyophilized to obtain dry material.

Determination of the Concentration and Degree of Polymerization of Oligo- and Poly-D-mannuronan. The molar concentration of monomeric units in the substrate was determined gravimetrically, and the concentration of reducing ends was determined by the bicinchoninic acid assay using sodium glucuronate to construct a standard curve. Briefly, the reducing end of oligo- and polysaccharides reduces Cu^{2+} to Cu^{+} at 60 °C, which in turn forms a complex with a chelator, bicinchoninic acid, under mild alkaline conditions (*17*). The average degree of polymerization of the sample is calculated as the concentration of monomeric units divided by the concentration of reducing ends.

Characterization of the Reaction Product. A large-scale reaction was conducted so sufficient product could be

produced for ^{1}H NMR analysis. A solution containing 0.5 mM poly-D-mannuronan ($dp_{average} = 93$), 20 mM Tris, 10 mM MES, 10 mM acetic acid, and 100 mM NaCl (pH 7.27) was prepared in a total volume of 9.8 mL. The reaction was initiated by the addition of 1 mg of C5-mannuronan epimerase in a volume of 0.2 mL. The solution was incubated at 25 °C in a water bath, and aliquots were removed after 2, 17, 28, 40, 48, 64, 73, 174, 318, and 663 h. The enzyme was precipitated from each aliquot that was removed by addition of a small volume of chloroform and removed from the sample by centrifugation. The supernatant was mixed with ethanol to precipitate alginate, and after centrifugation, the pellet was lyophilized overnight. The lyophilized samples were dissolved in D_2O and analyzed by ^{1}H NMR. All NMR experiments were performed on a Bruker DRX-500 spectrometer equipped with a temperature unit. Data collection was carried out at an elevated temperature (80 °C), and temperature stability was 0.1 °C. Calibration of the ^{1}H NMR spectrum at 80 °C was done by the addition of ethanol as an internal standard in a near-stoichiometric amount; the chemical shift of the methyl protons of ethanol (δ, 1.17 ppm), which is not sensitive to temperature, was compared to the signals of the analyte.

Ca^{2+} Dependence of the Epimerization Reaction. The C5-mannuronan epimerase reaction was assayed in the presence of several different Ca^{2+} concentrations; the amount of poly-D-mannuronan in each assay was adjusted such that the concentration of uncomplexed poly-D-mannuronan remained constant. The appropriate amounts of Ca^{2+} and poly-D-mannuronan for each assay were calculated using a value of 1 mM for the dissociation constant of the binary complex (*18*). It was assumed that each mannuronate residue in the polymer bound a single Ca^{2+} atom. The concentration of free poly-D-mannuronan was 0.5 mM, and the concentration of free calcium varied from 20 to 1400 μM.

Analysis of the Metal Ion Content of C5-Mannuronan Epimerase. The calcium content of purified, active C5-mannuronan epimerase was determined by flame atomic absorption spectroscopy using a model 200A spectrophotometer from Buck Scientific. The sample contained 50 μM C5-mannuronan epimerase from which the His tag had been removed. Liquid standards and samples, used in triplicate, were aspirated and mixed with acetylene and air. The light source was a calcium hollow cathode lamp with the wavelength set at 422.7 nm, and each absorbance reading was integrated over 7 s.

The concentration of other metal ions in the purified protein was determined by inductively coupled plasma-emission spectroscopy. The analysis was conducted at the University of Georgia with samples that contained 50 μM protein. Levels of Mg, Mn, Ca, Fe, Cu, and 20 other elements were measured.

pH−Rate Profiles. The pH dependence of the kinetic parameters for the reaction catalyzed by C5-mannuronan epimerase was determined by means of the continuous coupled assay as well as a fixed-time assay using poly-D-mannuronan that had an average degree of polymerization of 26. The pH was varied between 5 and 8.6 using a ternary buffer system composed of 20 mM Tris, 10 mM MES, and 10 mM acetic acid. The kinetic parameters k_{cat} and k_{cat}/K_m were fitted to eq 1

$$Y = \frac{C}{1 + 10^{3(pH-pK)}} \quad (1)$$

where Y is either k_{cat} or k_{cat}/K_m and C is the pH-independent value of Y.

RESULTS

Overexpression and Purification of C5-Mannuronan Epimerase. Recombinant C5-mannuronan epimerase was purified in 100 mg quantities through metal affinity chromatography. The purified protein was 99% homogeneous, based on Coomassie Blue-stained polyacrylamide gels. The solubility of the purified enzyme was improved by removal of the His_6 tag at the N-terminus, which was accomplished readily by thrombin-catalyzed cleavage. Removal of imidazole by stepwise dialysis against a buffer containing decreasing amounts of imidazole was found to mitigate protein precipitation. Enzyme stored at −80 °C in 10% (v/v) glycerol retained full catalytic activity for at least 2 months.

Metal Ion Dependence of the Epimerization Reaction. Investigation of the Ca^{2+} dependence of catalytic activity was first probed by determining the Ca^{2+} content of purified, active C5-mannuronan epimerase. Samples containing 50 μM protein contained 1.2 μM Ca^{2+}, as measured by atomic absorption spectroscopy, indicating that Ca^{2+} is not a tightly bound, stoichiometric component of the protein. Dialysis of the enzyme against EDTA did not cause a loss of activity. Furthermore, the catalytic activity of C5-mannuronan epimerase was unaffected by changes in the concentration of free Ca^{2+} (Figure 1A). Despite the broad range of free Ca^{2+} concentrations in the assays (1−1400 μM), initial velocities remained the same, 0.0095 s^{-1}, when 500 μM (total carbohydrate concentration) substrate was present. Calcium binds to poly-D-mannuronan (*18*), but the epimerase reaction was insensitive to the concentration of the complex, indicating that it cannot serve as a substrate (Figure 1B). Elemental analysis conducted by inductively coupled plasma-emission spectroscopy revealed that the purified protein contained no other metal ions above trace levels.

pH−Rate Profiles. The pH dependence of the kinetic parameters is shown in Figure 2. Both k_{cat} and k_{cat}/K_m decrease dramatically at alkaline pH, and the data were fitted to eq 1, which assumes that deprotonation of three ionizable groups with indistinguishable pK_a's leads to the decrease in k_{cat} and k_{cat}/K_m. The k_{cat} profile defined a pK_a value of 7.87 ± 0.05, and the k_{cat}/K_m profile defined a pK_a value of 7.6 ± 0.2. Alternative fits of the experimental data to functions describing ionization of two or four residues were considered less satisfactory on the basis of visual inspection of the data (Figure 2). The values of the kinetic parameters did not vary between pH 6 and 7.5; the reaction could not be characterized below pH 6 due to precipitation of the enzyme. Between pH 6 and 7.5, using poly-D-mannuronan that had an average dp of 26, the value for k_{cat} was 0.0218 s^{-1} and the value for K_m was 13 μM. The pH-independent value of k_{cat}/K_m was 1.7×10^3 M^{-1} s^{-1}, indicating that the reaction proceeds far more slowly than the rate of diffusional encounter.

Substrate Size Specificity. The kinetic parameters for the C5-mannuronan epimerase reaction were determined using pools of oligomannuronate in which the average dp varied from less than 10 to more than 100. As shown in Figure 3,

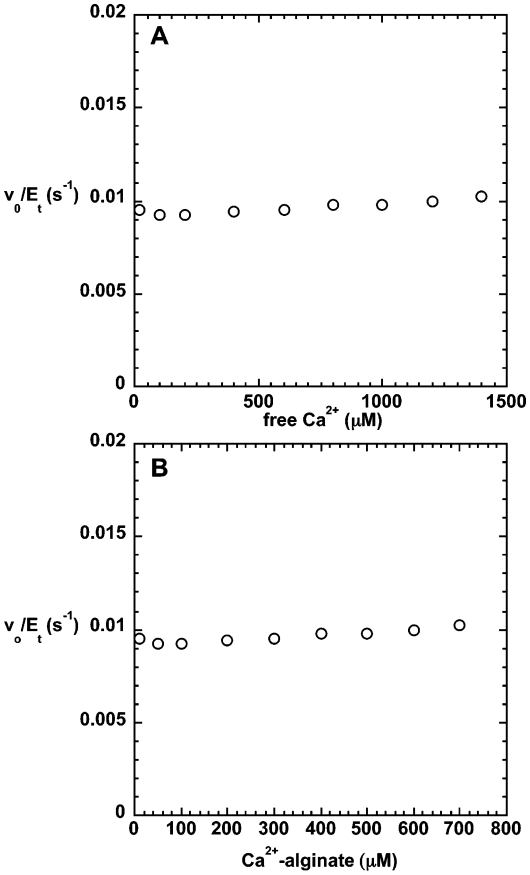


FIGURE 1: Variation of the steady-state rate with the concentration of the Ca^{2+} complex of poly-D-mannuronan (A) and free Ca^{2+} (B). The total carbohydrate concentration of the uncomplexed poly-D-mannuronan was 500 μM. Reactions were run at pH 7.27 and 25 °C.

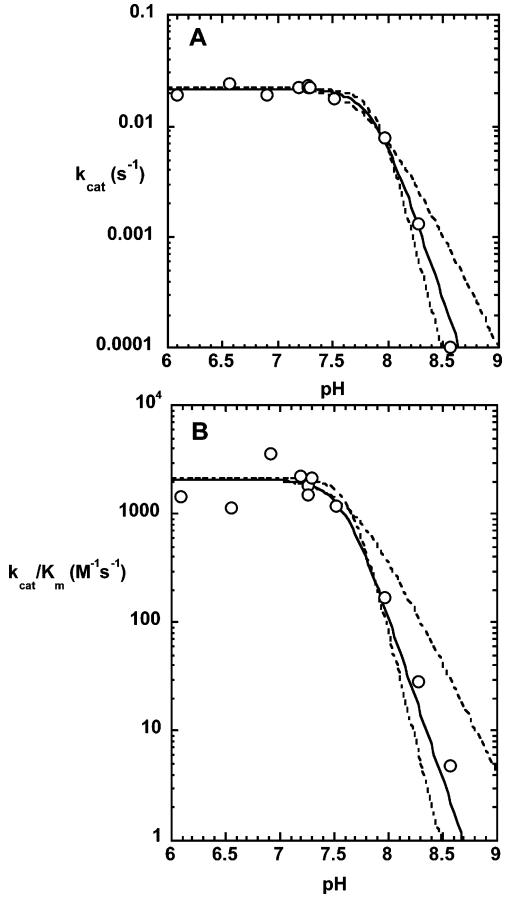


FIGURE 2: Kinetic parameters k_{cat} and k_{cat}/K_m for the epimerase reaction as a function of pH. The reactions were conducted as described in the text, and the solid lines show the fits to eq 1, which describes three ionizations. The dashed lines show alternative fits to equations describing two or four ionizations.

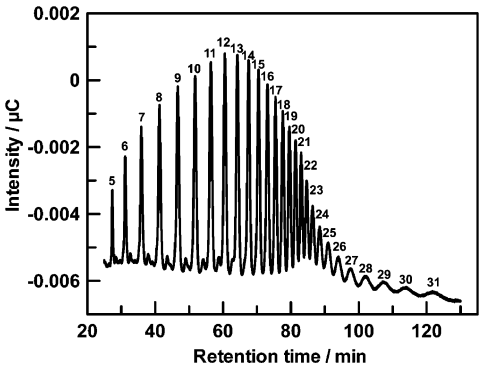


FIGURE 3: HPAE-PAD separation of oligo-D-mannuronans (dp = 1−31). The numbers above the eluting peaks represent the numbers of uronide residues of the individual oligomers.

high-performance anion exchange chromatography was used successfully to separate oligomannuronates up to a dp of ~30. The substrate pools for oligomannuronates with a dp of <30 that were used in the kinetic assays typically had a size variation of one to two units. The kinetic parameters as a function of substrate size are shown in Figure 4. K_m values decreased until they reached a plateau value of approximately 10 μM at a dp of 20. Similarly, k_{cat} values increased with increasing substrate size until a dp of 20, where they reached a maximum of 0.022 s^{-1}. No reaction could be detected for substrates that contained fewer than nine monomeric units.

Product Composition. C5-Mannuronan epimerase produces residues of α-L-guluronate within the substrate poly-β-D-mannuronan by inverting the configuration at C5 in the uronate monomeric unit. The composition of the product resulting from the action of C5-mannuronan epimerase can be determined by ^{1}H NMR analysis (*19*). Mannuronate residues and guluronate residues are readily distinguished by the chemical shifts of the proton on the anomeric carbon (4.6 ppm for mannuronate and 4.95 ppm for guluronate), so these signals provide a convenient means of monitoring the extent of the epimerization reaction. The chemical shift of the C5 proton of guluronate is exquisitely sensitive to the identity of neighboring residues, so the sequence of M and G residues in the polymer can be determined. The chemical shifts of the C5 proton for the underlined residue in the triads G<u>G</u>M, M<u>G</u>M, G<u>G</u>G, and M<u>G</u>G are 4.68, 4.65, 4.39, and 4.39 ppm, respectively. The C5 proton of mannuronate appears at 3.6 ppm and is not shown in Figure 5.

At very early stages of the reaction, C5-mannuronan epimerase forms heteropolymeric blocks of MGM sequence. However, as the reaction progresses, the enzyme displays a preference for acting on M residues that are adjacent to G residues. Indeed, beyond 25% conversion, only the G<u>G</u>G and M<u>G</u>G triads are populated. The enzyme is unable to convert every M residue to G; however, the reaction reaches an apparent equilibrium when the polymer contains 75% G.

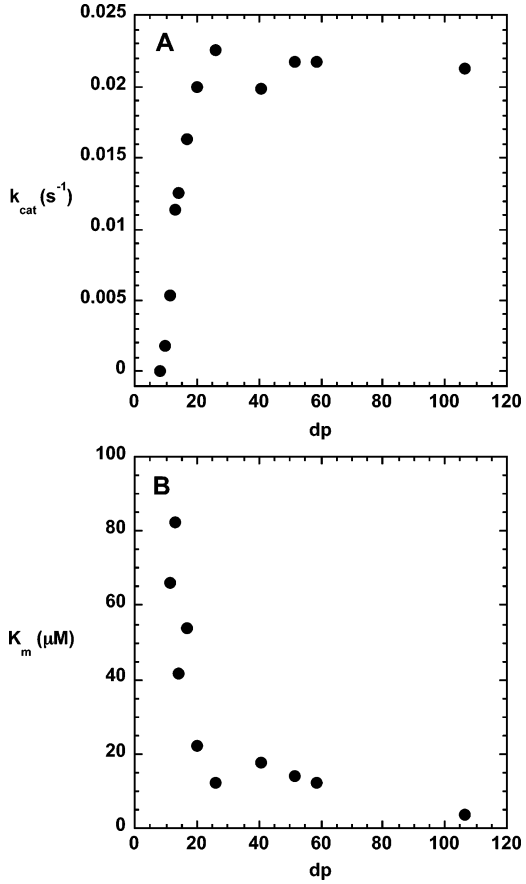


FIGURE 4: Substrate size specificity for the epimerase reaction. Variations in k_{cat} and K_m as a function of the degree of polymerization of oligo-D-mannuronan are shown. Epimerization was monitored by the continuous coupled assay at 25 °C and pH 7.27.

DISCUSSION

P. aeruginosa infections present severe risks to many patient populations; the pulmonary infections of cystic fibrosis patients are perhaps the most notorious (*1*). The presence of viscous phlegm in the lungs of cystic fibrosis patients is exacerbated by the bacterial production of alginate. The viscosity of alginate is a function of the composition of the polymer and the presence or absence of metal ions. Guluronate residues are introduced into the polymer via the action of C5-mannuronan epimerase; thus, in the context of developing strategies to treat *P. aeruginosa* infections, it becomes critical to characterize C5-mannuronan epimerase in detail.

The bacterium *A. vinelandii* produces a family of C5-mannuronan epimerases which catalyze the same reaction as the *P. aeruginosa* enzyme, but do not share significant sequence homology with it (*20*). The *A. vinelandii* enzymes are Ca^{2+}-dependent, and the initial studies of the *P. aeruginosa* epimerase included Ca^{2+} as a component of the assay solution (*7*, *11*−*13*). Several different roles for Ca^{2+} could be envisioned in the epimerase reaction. Since the epimerase reaction is likely to proceed via proton abstraction at the C5 position, Ca^{2+} could serve to stabilize the resulting carbanion in a fashion similar to that seen in the enolase reaction (*21*). The binding of Ca^{2+} to alginate has been well documented (*18*, *22*), so it is possible that the true substrate for the epimerase reaction is the Ca^{2+}−alginate complex. Finally, Ca^{2+} is known to be a tightly bound cofactor in some

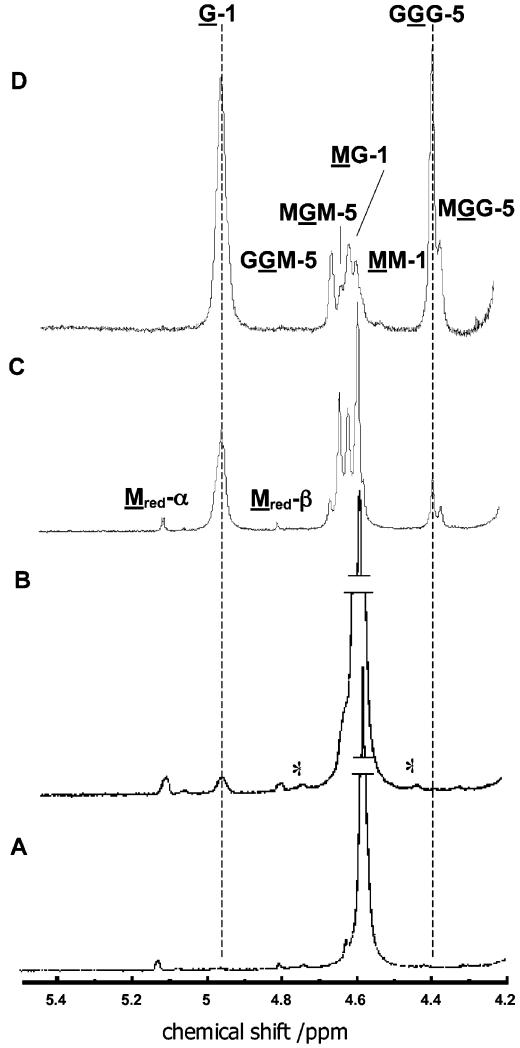


FIGURE 5: ^{1}H NMR analysis of alginate samples obtained after partial to exhaustive epimerization of poly-D-mannuronan by *P. aeruginosa* C5-mannuronan epimerase. Assay conditions are discussed in the text. Spectra were recorded at 500 MHz and 80 °C in D_2O. Peak assignments are given in panels C and D. Numbers designate the positions of the H-atom in the hexose ring; the underlined letters (M and G) denote the residues that are giving rise to the indicated peak, while the letters that are not underlined specify neighboring residues in the linear polymer. Peaks labeled with asterisks are due to ^{13}C coupling to MM-1 protons. (A) Spectrum of poly-D-mannuronan ($F_G = 0$, $F_M = 1$, $dp_{average} = 93$) used as the starting material in the assay. (B) Heteropolymeric alginate at a very low level of conversion ($F_G = 0.02$, $F_{GG} = 0$). (C) Heteropolymeric alginate at a moderate level of conversion ($F_G = 0.26$, $F_{GG} = 0.06$). (D) Heteropolymeric alginate formed as the final product by exhaustive epimerization by C5-mannuronan epimerase ($F_G = 0.75$, $F_{GG} = 0.50$). The vertical dashed line at 4.95 ppm marks the signal that arises from H1 on guluronate and serves to indicate the extent of the reaction. The vertical dashed line at 4.40 ppm marks the signal that arises from the C5 proton of the central guluronate residue in a GGG triad, and indicates the formation of G tracts.

enzymes (*23*, *24*). We therefore explored the Ca^{2+} dependence of the C5-mannuronan epimerase reaction.

The Ca^{2+} content of the purified, active enzyme was determined by atomic absorption spectroscopy. No Ca^{2+} above trace levels was observed, so Ca^{2+} is not a tightly bound cofactor in C5-mannuronan epimerase. Furthermore, dialysis of the purified enzyme against EDTA did not inactivate the enzyme.

The influence of Ca^{2+} on the rate of the epimerization reaction was determined using a continuous spectrophotometric assay. Initial experiments indicated that Ca^{2+} was slightly inhibitory; however, when the depletion of free polymannuronan from the solution by complexation with Ca^{2+} was taken into account (*18*), it was evident that Ca^{2+} has no effect on the C5-mannuronan epimerase reaction. As shown in Figure 1, free Ca^{2+} does not stimulate the reaction, nor is the Ca^{2+}−polymannuronan complex a substrate.

The absence of a requirement for Ca^{2+} in the catalytic reaction indicates that the presumptive carbanion intermediate in the catalytic reaction is not stabilized by a metal ion, as is the case in the reactions catalyzed by enolase superfamily members.

The substrate for C5-mannuronan epimerase in vivo is a polymer hundreds of residues long. Characterization of the size specificity of the enzyme can shed light on how large the area of interaction between the substrate and the enzyme is and, in a practical sense, can provide information about how large potential inhibitors should be. No reaction was detected with oligomers comprised of eight or fewer residues. The maximum velocity of the reaction increased dramatically between 10 and 20 residues, while the K_m for the substrate decreased over that size range. There was little variation in either kinetic parameter for substrates containing more than 20 residues (Figure 4). Using k_{cat}/K_m as a measure of substrate specificity, the oligosaccharide containing 20 residues was favored by more than 200-fold over the substrate containing only 10 residues.

If the K_m is treated as an approximation of the K_d for the substrates, it is apparent that the enzyme affinity increases with increasing substrate size up to a dp of 20. There is little change in K_m for substrates larger than 20 residues. The simplest interpretation of these data would suggest that C5-mannuronan epimerase interacts with the substrate over a surface that encompasses up to 20 sugar residues. This represents an extensive surface area; an oligosaccharide 20 residues in length would stretch approximately 100 Å. The AlgE2 and AlgE4 mannuronan epimerases from *A. vinelandii* exhibit increased activity with substrates up to 2000 residues in length (*25*), which corresponds to a size that is clearly too large to be accommodated by a single protein molecule. These data would seem to require that the substrate polymer serve as a nucleation center to which multiple molecules of the enzyme could bind. Experiments to explore whether this occurs with the *P. aeruginosa* epimerase are currently underway.

The value of k_{cat} increases for substrates with a dp between 10 and 20. Since k_{cat} is determined by extrapolating to conditions of infinite substrate, the decreased affinity of the enzyme for shorter substrates does not enter into the determination of k_{cat}. Thus, it is apparent that the increased binding energy that is available in larger oligosaccharides is used both to enhance the binding of the substrate to the enzyme in the ground state and to increase the rate acceleration in the enzyme−substrate complex. The rate acceleration can be quantitated through the relationship $\Delta\Delta G^{\ddagger} = -RT \ln(k_{20mer}/k_{10mer})$, where k_{20mer} is the k_{cat} for the reaction with the substrate containing 20 monomeric units and k_{10mer} is the k_{cat} for the substrate containing 10 monomeric units. Using the experimental values for k_{cat} obtained in this investigation, it can be calculated that the transition state is stabilized by an additional 1.5 kcal/mol for the dp = 20 substrate compared to the dp = 10 substrate. The data in Figure 4 indicate that k_{cat} increases in a roughly linear fashion for substrates with a dp between 10 and 20, suggesting that each additional residue beyond the first 10 contributes approximately 0.15 kcal/mol of binding energy to the rate acceleration.

The pH dependence of the kinetic parameters revealed that k_{cat} and k_{cat}/K_m both decreased dramatically at alkaline pH. Each pH profile described the ionization of three groups; because poly-D-mannuronan does not have any functional groups to which these ionizations could be assigned, they must arise from ionizable residues on the enzyme. Since the ionizations are observed in both the k_{cat} and k_{cat}/K_m profiles, the protonation state of these ionizable groups must be critical for catalysis (*26*).

The pH profiles are consistent with ionizations arising from residues that function as general acids in the catalytic reaction. The epimerization reaction requires abstraction of a proton from C5, followed by reprotonation from the opposite face of the intermediate. The ionizable residues observed in the pH profiles may be those that are serving as the general acids in the catalytic reaction. The data provide no evidence of the general bases that are expected to participate, but their absence from the pH profiles could simply mean that their pK_a's are below the experimentally accessible pH range. It is unclear why three ionizable residues would be required for catalysis, but a formal possibility that is consistent with the data is that the active site is able to act on more than one sugar residue at a time.

Alternatively, the ionizations may arise from residues that are not functioning as general acids in the catalytic reaction. Poly-D-mannuronan is a polyanion, and so it could reasonably be expected that interactions with cationic amino acid residues are important for binding. When a complex cannot form between the substrate and/or enzyme in the incorrect protonation state for reaction, the ionizations do not appear in the k_{cat} profile (*26*). Thus, the protonation states of the residues whose ionizations are evident in Figure 2 are not critical for formation of an enzyme−substrate complex, but their protonation state is critical for reaction. Rather than the ionizable residues being assigned to general acids at the active site, it seems more likely that they arise from residues that are important for binding of the substrate in a catalytically competent complex. Since polymannuronan is a long, rather uniform polymer which appears to interact with the enzyme over a relatively large surface area, we favor a model in which initial binding is relatively nonspecific but then the substrate is moved into proper register for reaction by electrostatic interactions with the correctly ionized residues.

The physical and chemical properties of alginate are determined by the mannuronate and guluronate content. Adjacent mannuronate residues are joined by diequatorial glycosidic bonds, which permit more rotational freedom, and thus more flexibility. Adjacent guluronate residues are joined by diaxial glycosidic bonds, which are more restrictive, and result in a stiff polymer with dense secondary structure. Accordingly, alginate that is composed primarily of G blocks has a lower solubility, and its aqueous solution has a higher viscosity than alginate containing M blocks; furthermore, G blocks form a rigid coordination polymer with divalent cations, such as calcium. Since C5-mannuronan epimerase

introduces guluronate residues into poly-D-mannuronan, the extent to which its reaction progresses is critical to the ultimate properties of the alginate.

Alginate that is isolated from mucoid *P. aeruginosa* colonies is characterized by the absence of stretches of adjacent guluronate residues (G blocks) (*27*). From this observation, it has been inferred that *P. aeruginosa* C5-mannuronan epimerase is unable to act on mannuronate residues that are adjacent to guluronate residues. The data in Figure 5 clearly belie this conclusion. It is apparent that GGG triads are readily formed; indeed, alginate that has been incubated with the epimerase exhaustively is composed of 75% guluronate and 25% mannuronate. Interestingly, the fraction of MG dyads (or GM dyads) is 0.5; therefore, the sequence of the final alginate product is best represented by repeating GGGM tetramers.

It is interesting that the end point of the reaction is reached when 75% of the uronate units are guluronates. The relative energies of mannuronate and guluronate are similar, so if the reaction were freely reversible, one would expect it to reach equilibrium when the polymer contained approximately equal amounts of mannuronate and guluronate. On the other hand, if the sugar ring conformation change that accompanies epimerization precludes binding to the enzyme, epimerization could be rendered irreversible, and one might expect the enzymatic reaction to continue until poly-G was formed. The observation that the reaction cannot proceed beyond incorporation of 75% guluronate residues suggests that the polymer composed of repeating GGGM tetrads assumes a conformation that cannot bind to the enzyme.

The apparent conflict between the composition of the alginate formed by C5-mannuronan epimerase in vitro and that isolated from mucoid cells can be resolved if the epimerization reaction takes place in competition with O-acetylation in vivo. Acetylated guluronate residues are not observed in alginate (*28*), so the extent of introduction of guluronate residues into the polymer will be controlled by the relative activities of the acetyltransferase and the epimerase.

The ^{1}H NMR data reveal another interesting feature of the C5-mannuronan epimerase reaction. Mannuronate residues adopt the $^4C_1$ conformation, but guluronate residues are found in the $^1C_4$ conformation, which allows the carboxyl group attached to C5 to assume the equatorial position in both sugars. The conformation change from a $^4C_1$ hexose to a $^1C_4$ hexose entails the movement of each equatorial substituent into an axial position, and vice versa. It is difficult to envision how this process could occur in the context of polymeric 1,4-linked sugar residues, in which one would expect there to be a significant kinetic barrier to the interconversion of the two chair forms.

One possibility is that the epimerization may occur via transient cleavage of the polymer. Such a mechanism has a number of attractive features. Stabilization of the carbanion generated by proton abstraction at C5 by formation of a glycal, 4-deoxy-L-*erythro*-hex-4-enopyranosyluronate, concomitant with cleavage of the 1,4-linkage would explain the absence of a metal requirement for the catalytic reaction. The transient generation of a cleaved intermediate would unlock the conformational restraint on the sugar unit undergoing epimerization and allow the intermediate to change conformation. Experiments are currently underway to examine the chemical mechanism of the epimerization reaction in more detail.

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