Chemical Mechanism and Specificity of the C5-Mannuronan Epimerase Reaction†

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ABSTRACT: C5-mannuronan epimerase catalyzes the formation of α-L-guluronate residues from β-D-mannuronate residues in the synthesis of the linear polysaccharide alginate. The reaction requires the abstraction of a proton from C5 of the residue undergoing epimerization followed by re-protonation on the opposite face. Rapid-mixing chemical quench experiments were conducted to determine the nature of the intermediate formed upon proton abstraction in the reaction catalyzed by the enzyme from *Pseudomonas aeruginosa*. Colorimetric and HPLC analysis of quenched samples indicated that shortened oligosaccharides containing an unsaturated sugar residue form as transient intermediates in the epimerization reaction. This suggests that the carbanion is stabilized by glycal formation, concomitant with cleavage of the glycosidic bond between the residue undergoing epimerization and the adjacent residue. The time dependence of glycal formation suggested that slow steps flank the chemical steps in the catalytic cycle. Solvent isotope effects on V and V/K were unity, consistent with a catalytic cycle in which chemistry is not rate-limiting. The specificity of the epimerase with regard to neighboring residues was examined, and it was determined that the enzyme showed no bias for mannuronate residues adjacent to guluronates versus those adjacent to mannuronates. Proton abstraction and sugar epimerization were irreversible. Existing guluronate residues already present in the polysaccharide were not converted to mannuronates, nor was incorporation of solvent deuterium into existing mannuronates observed.

Alginate is a linear copolymer of β-D-mannuronate and α-L-guluronate residues that are connected by 1 → 4 linkages. The opportunistic human pathogen *Pseudomonas aeruginosa* secretes alginate when it infects lung tissue in cystic fibrosis patients, and the viscous matrix it forms exacerbates the pulmonary difficulties suffered by the patient as a result of the defect in the cystic fibrosis transmembrane conductance regulator (*2*). *P. aeruginosa* infections are highly resistant to antibiotic treatment, and are the leading cause of morbidity and mortality in cystic fibrosis patients (*3*).

Alginate is synthesized as a linear homopolymer of β-D-mannuronate residues; inversion of configuration at C5 in some residues results in the introduction of α-L-guluronate residues. The epimerization is catalyzed by C5-mannuronan epimerase, a periplasmic enzyme that is encoded by the *algG* gene (*4*). The physical properties of alginate are determined by the relative amounts and distribution of uronate residues in the polymer. The composition of alginate can vary, not only in how much β-D-mannuronate and α-L-guluronate it contains, but also with respect to whether the two different uronic acids are distributed randomly throughout the polymer or localized to polymannuronate or polyguluronate tracts. Alginate that contains a high fraction of α-L-guluronate, particularly in a block structure of adjacent guluronates, binds Ca^{2+} in a cooperative manner and forms a stiff, dimeric chain. The Ca^{2+} binding and gelation properties of alginate vary with the sequence and amount of α-L-guluronate (*5*). In alginate that is produced by *P. aeruginosa*, some mannuronate residues are acetylated at O2 or O3; since guluronate residues are not acetylated, it appears that acetylated residues are not substrates for C5-mannuronan epimerase.

The polymer-level epimerization catalyzed by C5-mannuronan epimerase is a fascinating reaction. The reaction is chemically challenging to accomplish since it requires the abstraction of a nonacidic proton. Protons adjacent to carboxylic acids typically have pK_a's of 22−25, while those adjacent to carboxylates have pK_a's around 30 (*6*). It seems most likely that the carboxyl groups in alginate are ionized when the substrate binds to C5-mannuronan epimerase, so the C5 proton will certainly have a high pK_a. In contrast to many enzymes that catalyze abstraction of a proton adjacent to a carboxylate (*7*), C5-mannuronan epimerase does not utilize a divalent metal ion to stabilize the substrate anion (*8*).

Epimerization at C5 is accompanied by a conformational change in the pyranose ring. The β-D-mannuronate residues in polymannuronan adopt the $^4C_1$ conformation, but in alginate, the α-L-guluronate residues are in the $^1C_4$ conformation. The change in conformation allows the carboxylate to assume the equatorial position in both β-D-mannuronate and α-L-guluronate, and also has dramatic consequences for the shape of the polysaccharide (Scheme 1).

We have undertaken experiments to probe the mechanism of epimerization in the C5-mannuronan epimerase reaction. Transient-state kinetic studies demonstrate that the reaction proceeds via formation of an intermediate in which the glycosidic bond between adjacent residues is cleaved. Formation of a glycal intermediate provides a facile way to stabilize the anionic intermediate resulting from proton abstraction.

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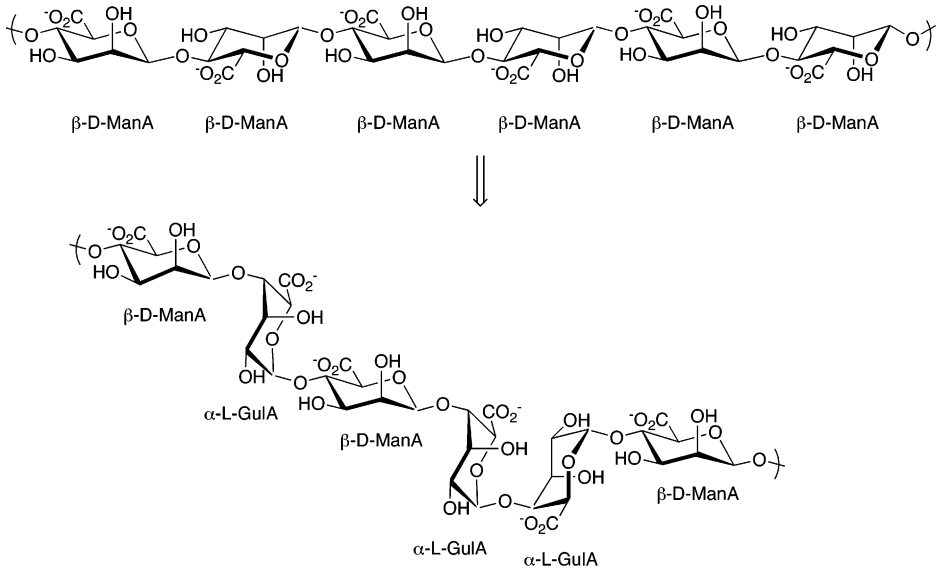
Scheme 1



MATERIALS AND METHODS

Recombinant C5-mannuronan epimerase was purified from a strain of *Escherichia coli* that harbored a plasmid derived from pET-14b containing the *algG* gene. Bacterial growth, protein expression, and purification followed the protocols established earlier (*8*). Aliquots of the purified enzyme were stored in buffer with 10% glycerol at −80 °C until use. Enzyme concentration was determined by absorbance measurements using an extinction coefficient at 280 nm of 69 840 M^{-1} cm^{-1}, which was calculated from the sequence of the mature protein by ProtParam (*9*).

Common biochemicals were obtained from Sigma/Aldrich Chemical Co. (St. Louis, MO), and buffers were obtained from Research Organics (Cleveland, OH). Routine assays of C5-mannuronan epimerase were conducted using a coupled enzymatic assay with L-guluronate lyase, which catalyzes the cleavage of glycosidic bonds between D-mannuronate and L-guluronate residues, with concomitant formation of a glycal residue that can be detected spectrophotometrically at 235 nm, or by a colorimetric assay (*8*). Oligomannuronate was purified from cultures of *P. aeruginosa* strain FRD462, which lacks a functional C5-mannuronan epimerase, and was the generous gift of D. E. Ohman. The procedures for oligomannuronate purification and fractionation based on size have been described (*8*).

Transient-State Kinetic Studies. Rapid-mixing chemical quench experiments were conducted using a Kintek RQF-3 instrument. C5-Mannuronan epimerase was dialyzed into buffer composed of 10 mM MOPS,[1] pH 6.8, 100 mM NaCl, 1 mM β-mercaptoethanol, 1 mM EDTA, and 10% (v/v) glycerol. Before use, the protein was concentrated to 48 mg/mL (875 μM). The substrate, poly-D-mannuronan, which was heterogeneous with respect to size, and had an average degree of polymerization of 60, was dissolved in 100 mM NaCl at a concentration of 1.22 mM.

Equal volumes of enzyme and substrate were mixed in the chemical-quench apparatus thermostated at 25 °C, and the reactions were terminated at fixed times by the addition of 100 mM acetic acid. The quenched samples were heated at 100 °C for 5 min, and the denatured protein was pelleted by centrifugation at 19 000*g*. Control experiments demonstrated that the quench conditions and subsequent workup did not induce cleavage of the oligosaccharide. In fact, partial hydrolysis of alginate requires incubation at 90 °C in 100 mM acetic acid for 2 days.

The amount of cleaved polymannuronan present in each quenched sample was determined by colorimetric assay for the glycal residue at the nonreducing end of the polysaccharide. A 50 μL aliquot of the quenched sample was mixed with 100 μL of 25 mM periodic acid. After thorough mixing, the solution was incubated at room temperature for 20 min. Excess periodic acid was reduced by the addition of 100 μL of 2% (w/v) sodium arsenite. Subsequently, 700 μL of 0.3% (w/v) thiobarbituric acid (pH 2.0) was added to the sample. The color was developed by heating the sample at 100 °C for 10 min; after cooling, the absorbance of each sample at 550 nm was measured.

A separate chemical quench experiment was conducted with substrate that was homogeneous with respect to size. Oligomannuronate, degree of polymerization (dp) 34, at a concentration of 24 μM, was mixed with 48 mg/mL epimerase; one sample was quenched after 10 ms, and a second was quenched after 5.5 s. To each quenched sample was added 25 μL of chloroform; after vigorous vortexing of the samples, the denatured protein was pelleted by centrifugation at 19 000*g*.

The uronic acids in the sample were analyzed by high-performance anion exchange chromatography using a Dionex DX-500 system equipped with a CarboPac PA-1 analytical column (4 × 250 mm) equilibrated in 100 mM NaOH and an ED40 electrochemical detector. Samples of 25 μL volume were injected onto the column, which was developed with a gradient from 0 to 1 M sodium acetate in 100 mM NaOH at a flow rate of 1.0 mL/min (*8*). The oligomannuronates were separated based on charge and detected by pulsed amperometry. The dp of the material in each peak was assigned

[1] Abbreviations: MOPS, 4-morpholinepropanesulfonic acid; dp, degree of polymerization, the 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.

by comparing the concentration of reducing ends to the concentration of monomeric units (*8*).

Characterization of the Reaction Product. The composition of the alginate produced by C5-mannuronan epimerase was evaluated as a function of the fractional conversion of M residues to G residues. The product of the enzymatic reaction was analyzed to determine whether the identity of neighboring residues affected the introduction of G residues.

Since conversion of polymannuronate to guluronate-rich alginate required extended incubation, all samples were prepared under sterile conditions using reagents that were filter-sterilized. A series of 1 mL total volume samples were prepared that each contained 0.1 mg C5-mannuronan epimerase, 2.1 mM oligomannuronan (average dp, 24), and 100 mM NaCl, in a ternary buffer composed of 20 mM Tris, 10 mM MES, and 10 mM acetate, pH 7.27. The samples were incubated at 25 °C for times ranging from 2.5 h to 27 days. Alginate produced by incubation with C5-mannuronan epimerase for 27 days was mixed with fresh enzyme and incubated until no further incorporation of G residues was observed. To terminate the reaction, 50 μL of $CHCl_3$ was added to the sample, which was vortexed for 2 min and centrifuged at 19 000*g* to pellet the denatured protein. The supernatant was decanted and mixed with an equal volume of ethanol to precipitate the alginate. The alginate was collected by centrifugation and lyophilized. The lyophilized sample was dissolved in D_2O and analyzed by ^1H NMR spectroscopy at 80 °C with a Bruker DRX-500 spectrometer. Product composition was determined as described previously (*8*).

The composition of alginate that would be formed through random conversion of M residues to G residues was determined using a Monte Carlo simulation. The Monte Carlo routine was written in PERL and allowed the user to input the length of the polymannuronan substrate and the number of iterations to perform. Each iteration consisted of the complete conversion of a poly-M chain to a poly-G chain, which required *N* steps, where *N* is the total length of the substrate.[2] Each step consisted of conversion of a randomly selected M residue to a G residue; the number of GG diads present in the polymer after each step was scored. The simulation output was the average number of GG diads that were counted at each step after all the iterations were completed.

Reaction Reversibility. The reversibility of the C5-mannuronan epimerase reaction was assessed by monitoring incorporation of solvent deuterium into the sample. A 1 mL sample was prepared that contained 10 mg alginate, (average dp, 25; F_G, 0.27; F_{GG}, 0.07), 100 mM NaCl, 10 mM sodium phosphate, pD 7.41, and 0.23 mg of C5-mannuronan epimerase in 95% (v/v) D_2O. The sample was incubated for 22 h at 25 °C; the reaction was terminated by the addition of 50 μL of $CHCl_3$ with vigorous vortexing, and the alginate was isolated for ^1H NMR spectroscopic analysis as described above.

Solvent Isotope Effect Studies. The steady-state kinetic parameters for the C5-mannuronan epimerase reaction were determined in H_2O and D_2O at pL 6.15. The reaction was monitored by enzymatic assay with L-guluronate lyase, as described (*8*). The reaction in H_2O was amended with 6% (v/v) glycerol to match the solution viscosity of the sample in D_2O. Reactions contained 10 mM MES, 100 mM NaCl, and oligomannuronate, average dp 30, at concentrations between 5 and 35 μM in a total volume of 1.0 mL. The catalytic reaction was initiated by the addition of 0.13 mg of C5-mannuronan epimerase. At fixed intervals between 1 and 60 min, 150 μL aliquots were removed and transferred to glass tubes that were placed in a sand bath at 170 °C for 10 s to terminate the reaction. The pH of the cooled samples was adjusted to 8.5 by the addition of 10 μL 1 M Tris base. Aliquots of 135 μL volume were removed and incubated with 3 μg of L-guluronate lyase at 25 °C for 2.5 h. The concentration of unsaturated uronate derived from L-guluronate lyase-catalyzed cleavage between M and G residues was determined from the absorbance of the sample at 235 nm. The initial velocity data were fitted to eq 1, which describes isotope effects on *V* and *V*/*K*. In eq 1, *A* is the substrate concentration, F_i is the fractional D_2O content of the solvent, $E_{V/K}$ is $^D(V/K) - 1$, and E_V is $^DV - 1$.

$$v = \frac{VA}{K(1 + F_iE_{V/K}) = A(1 = F_iE_V)} \quad (1)$$

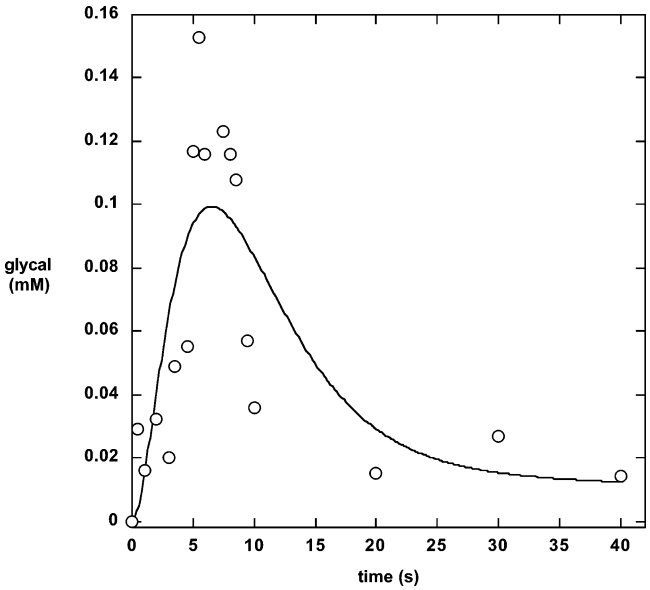


FIGURE 1: Time dependence of glycal formation in the C5-mannuronan epimerase reaction. The reaction was conducted with 0.4 mM enzyme and 0.6 mM oligomannuronan, as described in the text. The points are experimental, and the line is the result of a simulation of the following mechanism. E + oligoM_n → E·oligoM_n → E*·oligoM_n → E·oligoM_{n-1}G → E + oligoM_{n-1}G, where k_1 is 1.1 mM^{-1} s^{-1}; k_2 is 0.20 s^{-1}; k_3 is 0.31 s^{-1}; and k_4 is 0.014 s^{-1}. The simulation was conducted using the program DYNAFIT (*18*).

RESULTS

Detection of the Reaction Intermediate. Samples that were quenched with the rapid-mixing apparatus were assayed using the thiobarbituric acid assay, in which β-formylpyruvate is formed from the glycal residue at the nonreducing end of the oligosaccharide. The results are presented in Figure 1, which shows transient formation of a glycal intermediate. The maximum amount of glycal was observed approximately 5 s after mixing. From the known extinction coefficient of β-formylpyruvate, 29 000 M^{-1} cm^{-1} (*10*), the glycal con-

[2] The program is available from the authors on request.

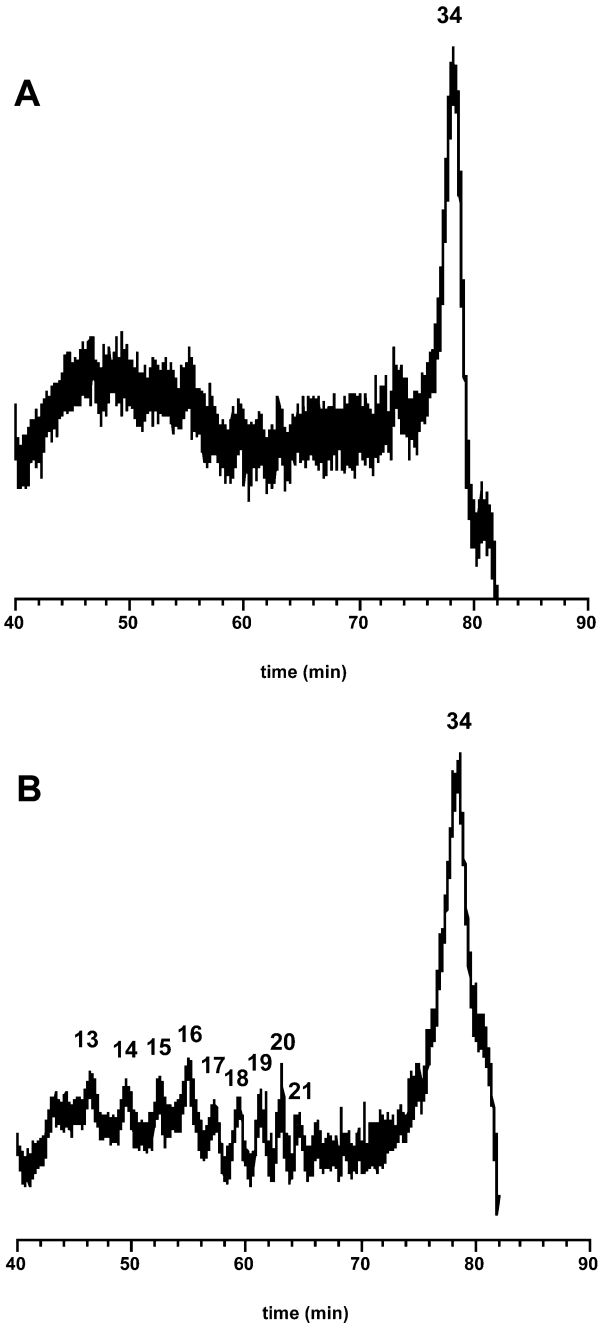


FIGURE 2: HPLC analysis of C5-mannuronan epimerase reaction. The samples were chromatographed on a CarboPac PA-1 column using the conditions discribed in the text. The oligosaccharides in the samples were separated based on charge, which is proportional to the number of residues in the oligosaccharide, and detected by pulsed amperometry. (A) Sample quenched after 10 ms. (B) Sample quenched after 5.5 s.

centration at each time point could be calculated, although the lack of precision in the data due to the volume changes that occur during the workup is evident.

To confirm that the glycal intermediate that was detected colorimetrically arose from transient cleavage of the substrate, a quenched sample was analyzed by anion exchange HPLC. Under the chromatographic conditions used, the oligosaccharides in the sample were separated according to size. HPLC analysis of a control sample that was quenched after 10 ms of reaction, where no glycal was detected colorimetrically, was homogeneous with respect to size. However, a sample that was quenched after 5.5 s, when

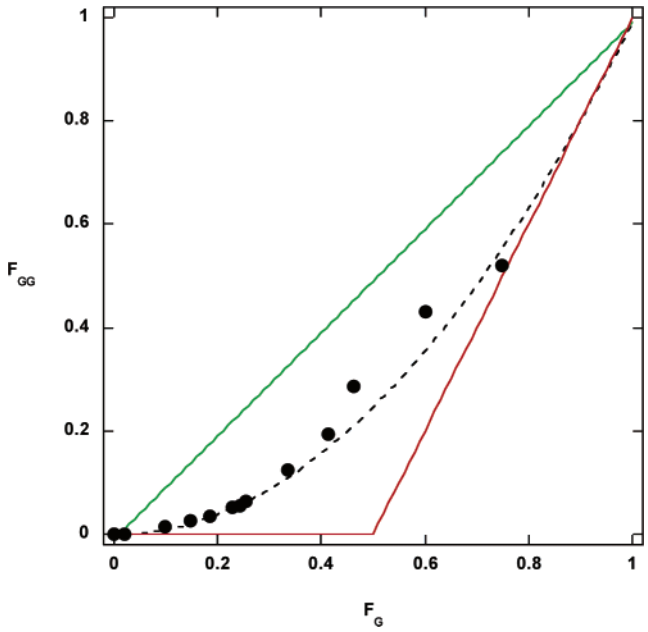


FIGURE 3: GG diad formation as a function of the extent of the C5-mannuronan epimerase reaction. The points are experimental, and the dashed line illustrates the expected curve for random conversion of M to G. The green line illustrates the pattern of incorporation that would be observed if the enzyme had complete specificity for acting on residues adjacent to G. The red line illustrates the pattern that would be observed if the enzyme exhibited specficity for M's that are adjacent to other M's.

glycal formation detected colorimetrically was at a maximum, contained a series of oligosaccharides, all smaller than the 34 residue substrate (Figure 2).

Positional Specificity of Epimerization. The incorporation of G residues into the oligomeric substrate as a function of the extent of the reaction was assessed by ^{1}H NMR spectroscopy, which allows adjacent G residues to be distinguished from G's that neighbor M residues. The fraction of GG diads as a function of G incorporation into the oligosaccharide is shown in Figure 3. The reaction reached an endpoint when the guluronate content of the product reached 75%. The results of the Monte Carlo simulation are also shown in Figure 3. The simulation was conducted for an oligomer 100 residues in length and was iterated 1000 times. The output from the simulation conformed to the relationship $F_{GG} = (F_G)^2$.

Reaction Reversibility. The C5-mannuronan epimerase reaction was conducted in the presence of D_2O in order to assess reversibility of the catalytic reaction. After 22 h of reaction, the guluronate content of the substrate increased from 0.27 to 0.33, while the fraction of GG diads remained constant at 0.07 (Figure 4). The composition of the alginate before and after the epimerization reaction was assessed by monitoring the intensities of the ^{1}H signals at 4.624, 4.600, 3.959, 3.841, 3.667, and 3.650 ppm, which arise from MG-1, MM-1, M-2, M-4, M-3, and M-5, respectively (*1*).[3] The intensity of the signal from M-5 was compared to that from M-3; the ratio of M-5/M-3 was 0.92 in the starting material and in the product.

[3] The nomenclature used by Larsen et al. is followed (*1*). The number designates the position of the proton on the hexose ring, and the underlined letter indicates the residue where the proton is located. The letters that are not underlined indicate neighboring residues.

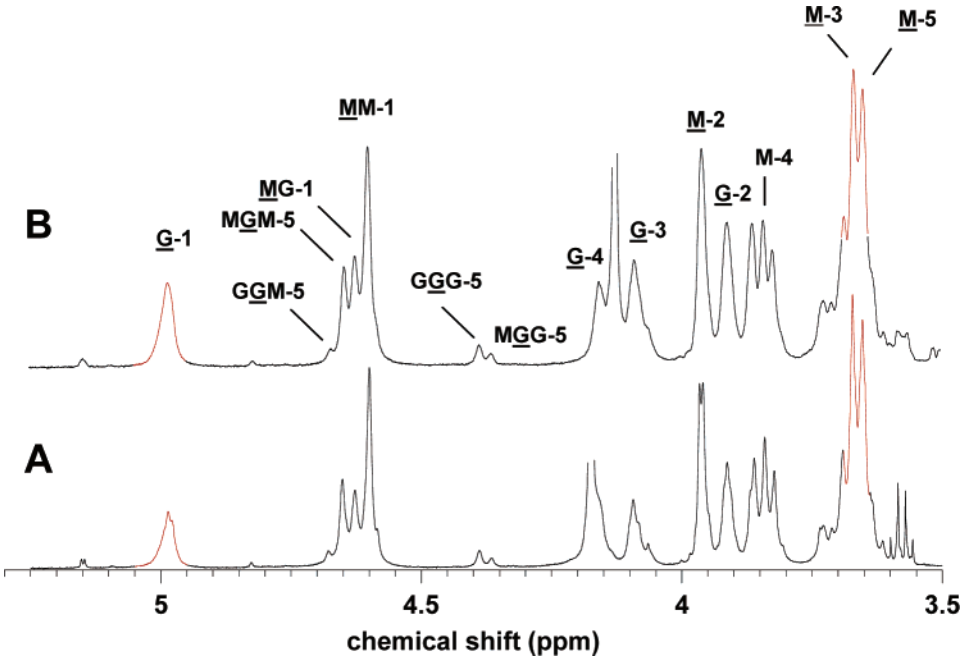


FIGURE 4: ^{1}H NMR spectra of alginate in 75% D_2O. (A) Starting material, 27% G. (B) Product, 33% G. The red portions of the spectra indicate the peaks that were monitored in order to determine whether the reaction was reversible.

Solvent Isotope Effects. There was no statistically significant solvent isotope effect on the C5-mannuronan epimerase reaction. The initial velocity kinetic data were fitted to eq 1, which yielded values of 1.1 ± 0.3 for $^{D2O}(V/K)$ and 1.2 ± 0.3 for ^{D2O}V.

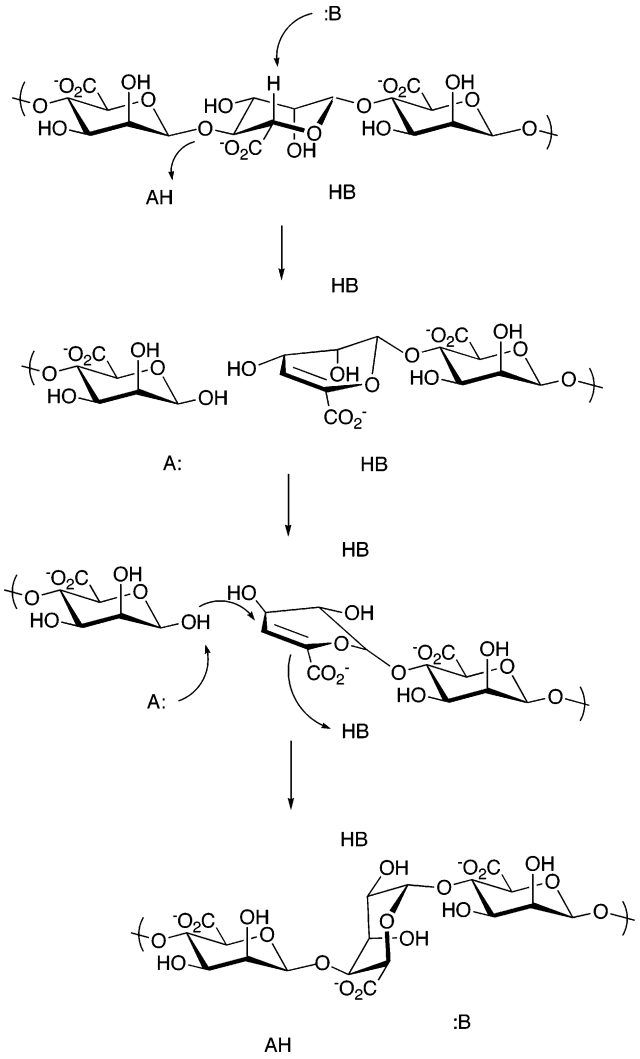
DISCUSSION

Epimerization at C5 of D-mannuronate to generate L-mannuronate is a key step in the biosynthesis of alginate. Rather than incorporate two different monosaccharides into the polysaccharide, alginate production proceeds by synthesis of a homopolymer, followed by epimerization at some residues within the polymer. The conversion of β-D-mannuronate to α-L-guluronate is accompanied by a conformational change in the pyranose unit, from $^{4}C_1$ to $^{1}C_4$.

Studies of C5-mannuronan epimerases from *Azotobacter vinelandii*, which are unrelated by sequence homology to the C5-mannuronan epimerase used in the present work, used ^{3}H-alginate as the substrate, and exchange of ^{3}H into solvent water was observed, establishing that abstraction of the C5 proton accompanied epimerization (*11*). The mechanistic possibilities for epimerization are defined by how the incipient carbanion is stabilized. Enzymes in the enolase superfamily, which catalyze the abstraction of a proton adjacent to a carboxylate, stabilize the negative charge that is delocalized onto the carboxylate group by coordination to a divalent metal ion (*7*). C5-Mannuronan epimerase does not contain a metal ion, nor is there a requirement for a metal ion for catalytic activity, so this mechanism does not appear to be operative (*8*).

A second possibility is that the reaction proceeds via β-elimination, followed by reformation of the glycosidic bond with protonation on the opposite face of the residue undergoing epimerization (Scheme 2). The biological cleavage of alginate occurs by β-elimination, not hydrolysis (*12*), suggesting that the low-energy pathway for epimerization would be stabilization of the leaving group alkoxide rather than the aci-acid functional group that would form by delocalization of the charge onto the carboxylate. Gacesa

Scheme 2



recognized this in 1987 and hypothesized that β-eliminations are a common feature of alginate lyase and alginate epimerase mechanisms (*13*).

An obvious prediction of the β-elimination mechanism is that a glycal intermediate forms transiently. This prediction is borne out by the data in Figure 1, which were obtained by rapidly mixing C5-mannuronan epimerase with a saturating concentration of polymannuronan, and quenching by addition of acid. The subsequent treatment of the samples with thiobarbituric acid allowed the detection of unsaturated saccharides at the nonreducing end of the substrate. There was no glycal present at the beginning of the reaction, but it could be detected within seconds. The glycal concentration peaked at around 5 s, and then decreased to a constant, low level, which presumably represents the concentration of intermediate bound to the enzyme at steady-state.

Another prediction of the β-elimination mechanism is that the substrate is transiently cleaved in the course of the epimerization reaction. We sought evidence that this occurs by examining the composition of a reaction mixture that was quenched after 5.5 s, when glycal formation that was detected colorimetrically was at a maximum. Substrate that was homogeneous with regard to size was used in this experiment in order to simplify the detection of shorter oligosaccharide intermediates. As shown in Figure 2, HPLC analysis of the reaction quenched after 5.5 s was composed of a family of shorter oligosaccharides. The control sample, that was quenched after 10 ms, before any appreciable reaction occurred, contained only the 34 residue substrate.

Although *P. aeruginosa* C5-mannuronan epimerase encoded by *algG* has little primary sequence homology with the family of C5-mannuronan epimerases encoded by the *algE* genes in *A. vinelandii*, the chemical mechanisms that they employ appear to be the same. The β-elimination mechanism proposed here is consistent with the finding that AlgE7 has both lyase and epimerase activity, and the elegant demonstration that a chimeric protein built from a domain of AlgE1 and a domain of AlgE7 is bifunctional (*14*).

It is noteworthy that the oligosaccharides detected in the quenched sample ranged in size from 13 to 21 residues in length. Pairwise combinations of the intermediate oligosaccharides can be constructed to yield a 34 residue oligosaccharide, corresponding to the size of the substrate and product. These data demonstrate that the enzyme generates only one cleavage site at a time on the substrate. It is also apparent that the epimerase does not bind near the end of the oligosaccharide, and there is no specificity for a cleavage site in the middle portion of the substrate. Perhaps this is not surprising; since the substrate for the first round of catalysis is a homopolymer, at a sufficient distance from the ends, there is nothing to distinguish one residue from another.

The product of the first round of catalysis is also the substrate for the next round, and after conversion of one mannuronate residue to guluronate, the substrate is no longer a homopolymer. Thus, there is the potential for sequence specificity in the epimerization reaction, and we sought to determine whether the enzyme exhibited a preference for acting on mannuronate residues that were adjacent to other mannuronate residues, a preference for acting on residues adjacent to guluronate residues, or whether there was no preference. Extended incubation of C5-mannuronan epimerase with polymannuronan results in the formation of alginate that contains 75% guluronate residues. During the conversion, the reaction mixture was sampled periodically and the fractional conversion of M residues to G residues and the amount of GG diads present were determined by ^{1}H NMR spectroscopy. The experimental data were compared to the output from a Monte Carlo simulation that was conducted to determine the GG diad content of an alginate polymer that was formed by random conversion of M residues to G. The envelope of possible outcomes for sequential conversion of a poly-M chain to poly-G is also indicated in Figure 3. The green line shows the relationship between F_G and F_{GG} that would be observed if the epimerase showed an absolute specificity for acting on M residues that are adjacent to existing G's. In such a case, the epimerization reaction would proceed like falling dominoes from one end of the molecule to the other. The other extreme would be specificity for M residues that are not neighboring G's. In this case, the reaction would proceed to 50% conversion of M to G by acting on alternate M residues; each subsequent conversion of M to G would create two GG diads (GMG converted to GGG).

The experimental data obtained for the C5-mannuronan epimerase reaction fall on the line determined by random incorporation of G residues. Thus, it appears that the enzyme does not exhibit any neighboring residue specificity at early stages of the reaction. This result is somewhat surprising in light of the fact that poly-M and poly-G polymers, as well as heteropolymers of M and G, have quite distinct conformations. It is difficult to reconcile the lack of neighboring residue specificity with the inability of the enzyme to convert more than 75% of the residues to G. It remains unclear why the epimerization reaction cannot proceed to 100% conversion of M to G. One possibility is that a structural transition occurs in substrate containing more than 75% G, such that it can no longer bind to the enzyme.

A second possibility is that the reaction has reached thermodynamic equilibrium at 75% conversion of M to G. In molecules with a single chiral center, the equilibrium constant for racemization is unity, but that need not be the case in more complex molecules. In alginate, the effect of epimerization at C5 of one residue has repercussions beyond that residue, because guluronate residues adopt a different conformation than mannuronate, which in turn influences the shape of the polymer and its ionic and hydrogen-bonding interactions. If the substrate containing 75% guluronate represented the equilibrium mixture for the epimerase reaction, one would expect the conversion of M to G residues to continue, but at the same rate that G residues were converted to M's. Reaction reversal should be detectable based on the incorporation of solvent deuterium into C5 of mannuronate residues that would accompany epimerization of guluronate residues. However, when the reaction was conducted in D_2O using substrate that initially contained 27% G, no deuterium incorporation into mannuronate was detected. This observation indicates that the epimerase is unable to convert guluronate residues to mannuronate. Furthermore, proton abstraction from mannuronate residues is essentially irreversible. (If that were not the case, the ^{1}H NMR signal from the C5 proton of mannuronate would decrease in intensity faster than the rate of conversion of M to G; this was not observed.)

The irreversibility of proton abstraction implies a large forward commitment to catalysis, and the time course for glycal production shown in Figure 1 also indicates that chemistry is not rate-limiting in the overall reaction. The time course indicates a complex kinetic mechanism which we are able to evaluate qualitatively, but not quantitatively. It is clear that an intermediate accumulates, which indicates that a slow step occurs after its formation. Attempts to fit the data to a simple two-step sequential mechanism, A → B → C, where B is detected, yielded unsatisfactory results, and it became apparent that the time course, which exhibits maximal intermediate formation only after several seconds, could arise only if the kinetic mechanism also included a slow step that precedes intermediate formation as well. Figure 1 shows a simulated time course for a mechanism in which intermediate formation is flanked by slow steps. There are too many steps in the mechanism and too much noise in the data to obtain a unique, well-defined fit, but the simulation provides insight into the essential features of the kinetic mechanism.

An independent evaluation of whether the chemistry is rate-limiting in the epimerase reaction can be gained from measurement of solvent isotope effects. When the reaction is conducted in D_2O, protonation of the glycal intermediate will give rise to a kinetic isotope effect if the protonation step contributes to the rate-limiting portion of the reaction. No statistically significant solvent isotope effect on V or V/K was measured.

The transient-state kinetic data and solvent isotope effect measurements are consistent with a mechanism in which initial binding of the polymeric substrate to the enzyme is followed by a slow step that aligns the substrate more precisely for reaction. The pH dependence of the kinetic parameters also suggested a step in the reaction where the substrate was moved into register with active site residues before catalysis took place (*8*). Release of the product also is slow, and perhaps entails a reversal of the movements that occur upon substrate binding.

Alginate biosynthesis is a complex process that begins with synthesis of the polymannuronan precursors in the cytoplasm, continues in the periplasm, and ends with the secretion of the product out of the cell. It is believed that polymannuronan synthesis is catalyzed by proteins that are tightly associated with the inner membrane (*15*). C5-Mannuronan epimerase is periplasmic and appears to be associated with other proteins that have lyase and acetyltransferase activities (*16*, *17*). A fascinating topic for future investigation is the determination of how these protein−protein interactions affect epimerase activity.

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