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Functional Characterization of AlgL, an Alginate Lyase from *Pseudomonas aeruginosa*

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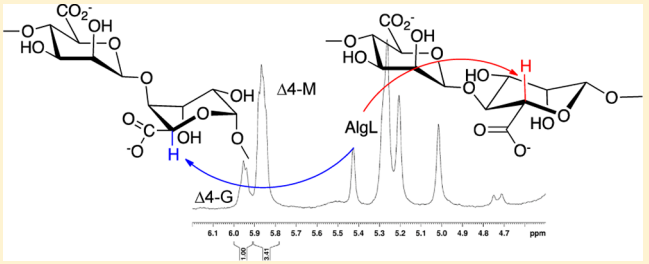
ABSTRACT: Alginate lyase (AlgL) catalyzes the cleavage of the polysaccharide alginate through a β-elimination reaction. In *Pseudomonas aeruginosa*, *algL* is part of the alginate biosynthetic operon, and although it is required for alginate biosynthesis, it is not clear why. Steady-state kinetic studies were performed to characterize its substrate specificity and revealed that AlgL operates preferentially on nonacetylated alginate or its precursor mannuronan. Mature alginate is secreted as a partially acetylated polysaccharide, so this observation is consistent with suggestions that AlgL serves to degrade mislocalized alginate that is trapped in the periplasmic space. The k_{cat}/K_m for the reaction increased linearly with the number of residues in the substrate, from 2.1×10^5 M^{-1} s^{-1} for the substrate containing 16 residues to 7.9×10^6 M^{-1} s^{-1} for the substrate with 280 residues. Over the same substrate size range, k_{cat} varied between 10 and 30 s^{-1}. The variation in k_{cat}/K_m with substrate length suggests that AlgL operates in a processive manner. AlgL displayed a surprising lack of stereospecificity, in that it was able to catalyze cleavage adjacent to either mannuronate or guluronate residues in alginate. Thus, the enzyme is able to remove the C5 proton from both mannuronate and guluronate, which are C5 epimers. Exhaustive digestion of alginate by AlgL generated dimeric and trimeric products, which were characterized by ^{1}H nuclear magnetic resonance spectroscopy and mass spectrometry. Rapid-mixing chemical quench studies revealed that there was no lag in dimer or trimer production, indicating that AlgL operates as an exopolysaccharide lyase.



Alginate is a linear polysaccharide that is secreted by *Pseudomonas aeruginosa* in response to various environmental stimuli and is responsible for the mucoid phenotype exhibited by the bacteria when they infect the lungs of cystic fibrosis patients. The onset of mucoidy in the lungs correlates with declining prognosis for survival for those patients,[1] and alginate production has been shown to promote bacterial persistence.[2]

The pathway for alginate synthesis from fructose 6-phosphate has been described, and the enzymes required for synthesis of the sugar−nucleotide precursor have been characterized biochemically.[3−5] Many questions remain about the final stages of synthesis and secretion, although structural studies of the proteins involved and extensive microbiological studies have provided a wealth of information (reviewed in ref 6). The chemical steps that occur in the latter stages of alginate biosynthesis are shown in Scheme 1. The first polymeric species in the pathway is mannuronan, a homopolymer of β-(1→4)-D-mannuronic acid, which is formed from GDP-mannuronic acid that is present in the cytoplasm. Mannuronan formation requires Alg8 and Alg44, which are associated with the inner membrane. The newly synthesized mannuronan is found in the periplasmic space where the final steps in synthesis occur. AlgG catalyzes epimerization of some residues to form α-L-guluronic acid, and AlgF, -J, and -I are required for acetylation of some mannuronic acid hydroxyl groups at C2 and C3. Somewhat paradoxically, functional alginate biosynthesis requires AlgL, which is a periplasmic alginate lyase. Deletion of *algL* is lethal, and microscopic examination of the cells reveals that alginate or a precursor accumulates in the periplasmic space until the cells burst.[7]

Some features of the AlgL reaction have been reported,[8] but the mechanism has not been examined in detail. Interest in alginate lyase stems not only from its involvement in the synthesis of alginate, a virulence factor that is important for the establishment of chronic lung infections, but also from the compelling nature of its catalytic reaction. The reaction is a β-elimination requiring abstraction of the C5 proton, which is adjacent to a carboxylate, and therefore is extremely nonacidic. The specificity of AlgL has not been examined before and is interesting because the epimers mannuronate and guluronate adopt different conformations, so that the C5 proton in the two sugars is unlikely to occupy the same position with respect to catalytic residues at the active site. In this study, we have determined the substrate specificity and product distribution of the AlgL reaction.

MATERIALS AND METHODS

Laboratory reagents were purchased from Sigma-Aldrich unless otherwise indicated and used without further purification. MES was obtained from Research Organics.

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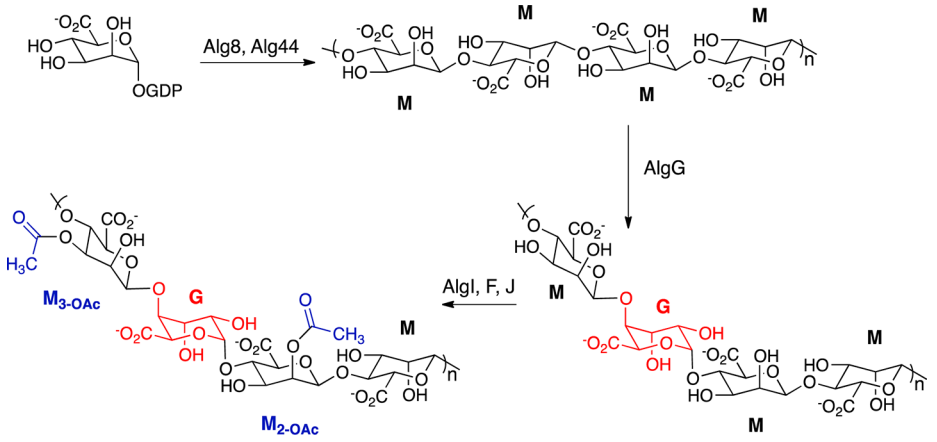
Scheme 1



Purification of AlgL. Recombinant AlgL with a His_6 tag at the C-terminus was purified from *Escherichia coli* cells harboring an expression plasmid that was constructed by inserting the *algL* coding sequence into pET-26b using the NcoI and XhoI restriction sites.[9] The construct included a periplasmic-localizing pelB leader sequence, which was cleaved during translation. Transformed *E. coli* C41(DE3) cells were selected by growth on plates with kanamycin. For production of AlgL, cells were grown in LB medium supplemented with kanamycin (50 μg/mL) at 37 °C with rotary shaking until the OD_{600} reached 0.6−0.8. Subsequently, 1 mM IPTG was added to induce expression of AlgL, and growth was continued at 25 °C for 16 h. Cells were harvested by centrifugation at 6000*g* for 15 min, yielding 3−4 g of cell paste/L of medium.

The purification procedure was based on a modification of the published protocol.[9] In a typical purification, 6 g of cell paste was suspended in 20 mL of buffer composed of 50 mM Tris (pH 7), 500 mM NaCl, 2 mM DTT, 2% glycerol, and 5 mM imidazole. Before lysis, 250 units of benzonase (Novagen) and 1 mM PMSF were added. Cells were lysed by being passed through a French press four times. Insoluble cell debris was removed by centrifugation at 12200*g* for 45 min. The cell-free extract was loaded onto a 5 mL His Trap HP (GE) affinity column charged with Co^{2+}. The column was washed with 30 mL of resuspension buffer and then 30 mL of resuspension buffer containing 20 mM imidazole. AlgL was eluted from the column with 30 mL of resuspension buffer containing 200 mM imidazole. AlgL-containing fractions were combined and dialyzed against fresh resuspension buffer and then chromatographed on the same column a second time under the same conditions. The purity of the final product was checked by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), and the sample was concentrated to >2 mg/mL using spin concentrators (Pierce). The enzyme concentration was determined by absorbance measurements using an extinction coefficient at 280 nm of 67380 M^{-1} cm^{-1}, which was calculated from the sequence of the mature protein by ProtParam.[10] Glycerol was added to the protein to a final concentration of 10% (v/v), and aliquots were stored at −80 °C.

Substrate Isolation and Characterization. PolyM was isolated from *P. aeruginosa* strain FRD462, which contains a point mutation in *algG* and so does not have mannuronan epimerase activity.[11] Alginate was isolated from overproducing *P. aeruginosa* strains PDO486, which was the generous gift of D. Ohman (Virginia Commonwealth University, Richmond, VA), and PDO300. In each case, the appropriate strain was grown overnight in liquid LB medium at 37 °C. Aliquots were then spread on solid medium that contained 22.5 g of Pseudomonas Isolation Agar (Remel), 12.5 g of glycerol, 12.5 g of LB broth, 37.5 g of sucrose, and 7.5 g of agar, per liter. Inoculated plates were incubated at 28 °C for 3 days. The viscous mass was scraped from the surface of the plates, suspended in 0.85% NaCl, and stirred at room temperature until a homogeneous suspension was obtained. The cells were removed by centrifugation at 1700*g* for 15 min. An equal volume of 2-propanol was added to the supernatant to precipitate the polysaccharide. The polysaccharide was dissolved and precipitated with 2-propanol two more times, first from 0.85% (w/v) NaCl and then from 1 M NaCl. The collected polysaccharide was redissolved in 0.85% NaCl, incubated with 5−10 mg of Pronase for 1 h, and reprecipitated with 2-propanol. Deacetylated alginate and polyM were obtained by incubating dissolved samples in 0.1 M NaOH for 30 min at room temperature, followed by reprecipitation with 2-propanol. To obtain oligosaccharides of various lengths, samples were subjected to acid hydrolysis. Samples were refluxed at pH 5.6 for 1 h and then at pH 3.5 for a variable length of time (0−60 min) to generate samples containing ∼10−250 monomeric units. The uronic acid content was quantified by the carbazole assay as described previously.[12] The reducing end concentration was quantified with 3-methyl-2-benzothiazolinonehydrazone as described previously,[13] and the polymer length (dp) was calculated as the ratio of monomeric units to reducing ends.

The composition of the isolated material was determined by ^1H NMR as described previously.[14] Samples were subjected to partial acid hydrolysis to decrease the polymer size so that well-resolved signals could be obtained. Lyophilized samples were dissolved in D_2O, and spectra were recorded at 80 °C using solvent suppression with a Bruker DRX 300 MHz spectrometer.

AlgL Assay. Product formation was detected by the increase in absorbance at 240 nm arising from formation of unsaturated uronates. Assays were conducted at 25 °C in a Carey 50 Bio UV−vis spectrophotometer. An extinction coefficient of 6150 M^{-1} cm^{-1}[15] was used to convert absorbance to product concentration. The reaction buffer contained 100 mM sodium phosphate, 100 mM MES, 100 mM Tris, and 200 mM NaCl

(pH 7.1) unless otherwise indicated. Initial velocity kinetics data were fit to the Michaelis–Menten equation.

Purification of Dimers and Trimers. Dimers and trimers obtained from complete AlgL digestion of polyM or alginate were purified using a DEAE column prior to HPLC analysis. Reaction mixtures containing up to 20 mg of digested polysaccharides were loaded onto a DEAE-Sephadex A-25 column (2.8 cm × 19 cm), which was equilibrated with 25 mM ammonium acetate pH 6.8. A gradient from the equilibration buffer to 1 M NH_4OAc over 1 L with a flow rate of 5 mL/min was used to elute the oligosaccharides. The separation was conducted at room temperature. Fractions containing AlgL digestion products were identified from their absorbance at 240 nm.

Dimers and trimers for mass spectral analysis were purified by size exclusion chromatography. Reaction mixtures containing up to 10 mg of digested polysaccharides were loaded onto a Bio-Gel P-2 column [1.5 cm × 28 cm (Bio-Rad)] equilibrated with 1 mM phosphate (pH 6.8). The column was developed at 0.5 mL/min, and fractions containing AlgL digestion products were pooled and concentrated by rotary evaporation. Because MES from the AlgL stock solution was found to coelute with reaction products and interfere with MS analysis, AlgL digestions of samples for MS analysis were conducted without MES.

HPLC Characterization. Samples were dissolved in 0.1 mL of 100 mM NaOH containing 1 mM saccharate as an internal standard and injected onto a CarboPac PA-100 column (4 mm × 250 mm) using a Dionex DX-500 HPLC system equipped with an ED40 electrochemical detector. The solvent flow rate was 1 mL/min, and products were eluted using the following gradient: 0% B from 0 to 10 min, gradient to 60% B from 10 to 24 min, gradient to 80% B from 24 to 40 min, and gradient to 100% B from 40 to 50 min (solvent A was 0.1 M NaOH and solvent B was 1 M sodium acetate in 0.1 M NaOH). Under these conditions, saccharate eluted at 22 min, the dimeric product at 25 min, and the trimer at 28 min.

Chemical Characterization of Products. ESI-MS was performed on a Thermo-Finnigan TSQ7000 triple-quadrupole MS instrument with the API2 source and Performance Pack (ThermoFinnigan, San Jose, CA). Oligosaccharides were desalted by size exclusion chromatography with a P-2 column as described above and delivered to the electrospray source using a syringe pump at a flow rate of 10 μL/min. The MS instrument was run in negative mode, with the mass scan range from 100 to 1500 Da. The capillary temperature was kept at 250 °C, and the electrospray needle voltage was 4.5 kV. Nitrogen sheath gas was provided to the source from a nearby dewar of liquid nitrogen at 40–80 psi. Oligosaccharides identified by ESI-MS were further confirmed by MS/MS on the same instrument under the same conditions except that argon was used as the CID gas. Purified products from the AlgL reaction were characterized by ^1H NMR with a Bruker DRX 300 MHz spectrometer. Samples containing 0.5–1 mg of product were dissolved in D_2O; spectra were obtained at 60 and 90 °C using a pulse sequence that suppressed the HOD peak.

Transient-State Kinetic Studies. Rapid-mixing chemical quench experiments were conducted using a Kintek RQF-3 instrument. PolyM was dissolved in 25 mM Tris, 25 mM sodium phosphate, 25 mM MES, and 50 mM NaCl (pH 7) at a final concentration of 2.5 mg/mL. The average dp was 71. AlgL was used at a concentration of 8.4 mg/mL. Equal volumes of polyM and AlgL were mixed in the chemical-quench apparatus thermostated at 25 °C, and the reactions were terminated at fixed times ranging from 20 to 250 ms by the addition of 100 mM HCl. For each time point, 12 individual samples were pooled; the pooled samples were neutralized by addition of NaOH, lyophilized, and dissolved in 100 μL of 100 mM NaOH with 1 mM internal standard (saccharate). Two to three replicate runs of each sample (25 μL) were analyzed by HPLC as described above.

RESULTS

Expression and Purification of AlgL. Robust overexpression of AlgL was observed, and purification using metal ion affinity chromatography yielded a protein that was >95% pure, based on Coomassie-stained SDS–PAGE. Higher yields of AlgL were obtained using Co^{2+}-NTA columns instead of Ni^{2+}-NTA columns, which were used in previous work[9] (data not shown). Samples supplemented with 10% (v/v) glycerol and stored at −80 °C were stable for several months. Although purified AlgL was stable and active, with no observable activity decrease even after storage at 4 °C for 2 weeks, its activity was observed to vary dramatically with protein concentration (Figure 1). Initiating the reaction by addition of substrate after allowing the enzyme to incubate in the assay solution for up to 30 min had little effect on the measured velocity. These results suggest that AlgL aggregates in a nonreversible or slowly reversible manner, but this has not been investigated further.

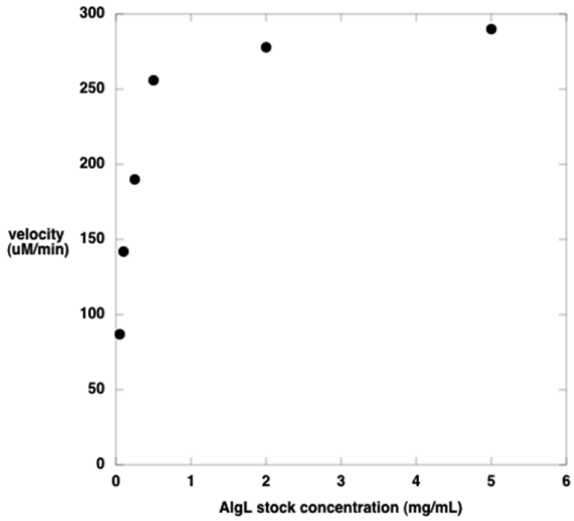


Figure 1. Activity of AlgL as a function of protein stock concentration. The polyM concentration was 20 μM, and polyM had an average dp of 34. Each reaction was initiated by the addition of 30 μg of AlgL from a stock solution at the concentration indicated.

In characterizing the AlgL reaction, we sought to determine characteristics that would speak to its functional role in the bacterium. Thus, we examined the dependence of the reaction on the length of the substrate and whether the enzyme exhibited specificity for acetylated or deacetylated substrates, because exogenous alginate encountered by the bacteria would be long, acetylated material. To gain insight into the catalytic mechanism of AlgL, we examined its specificity for cleavage at mannuronate versus guluronate residues and whether it acted as an exo or endo lyase.

Acetylated versus Deacetylated Substrates. Four potential substrates for AlgL were tested: alginate,[a] polyM,

acetylated alginate, and acetylated polyM. The kinetic parameters were determined by monitoring product formation with the spectrophotometric assay described above (Table 1).

Table 1. Steady-State Kinetic Parameters for the AlgL Reaction

substrate[a]	k_{cat}/K_m (s^{-1} M^{-1})	k_{cat} (s^{-1})	K_m (μM)
polyM	$(2.5 \pm 0.6) \times 10^6$	32 ± 3	13 ± 3
polyM-OAc	$(0.30 \pm 0.06) \times 10^6$	1.5 ± 0.1	5 ± 1
alginate	$(3.1 \pm 0.9) \times 10^6$	32 ± 4	11 ± 3
alginate-OAc	$(0.5 \pm 0.1) \times 10^6$	1.2 ± 0.1	2.6 ± 0.7

[a]PolyM and polyM-OAc used in the experiment had dp values of 133; alginate and alginate-OAc had dp values of 263.

Because the kinetic parameters vary with the number of sugar residues in the polymer, comparisons between acetylated and deacetylated substrates were made using substates containing the same number of residues. Because the k_{cat}/K_m values for deacetylated substrates are 10-fold higher than for the corresponding acetylated substrates, it appears that AlgL has been optimized for acting on unacetylated alginate or polyM.

Size Specificity. The steady-state kinetic parameters for cleavage of various sizes of polyM were determined (Figure 2). There was only modest variation in k_{cat} across the size range examined; however, K_m decreased with an increase in substrate length, and k_{cat}/K_m increased. Oligomers containing fewer than 9 or 10 residues were not substrates for AlgL or reacted too slowly to permit characterization at the concentrations used.

Cleavage at Mannuronate versus Guluronate. Exhaustive incubation of AlgL with alginate or polyM resulted in formation of dimeric and trimeric products. The products were characterized by ^{1}H NMR and mass spectrometry. Mass spectra revealed peaks consistent with trimers and dimers containing an unsaturated residue (m/z 527 and 351, respectively) as well as the monosodiated and disodiated trimers. MS/MS of each of these species revealed fragmentation consistent with mannuronate or oligomannuronan. The ^{1}H NMR spectra matched published spectra for dimers and trimers of mannuronate and guluronate containing the 4,5-unsaturated residues at the

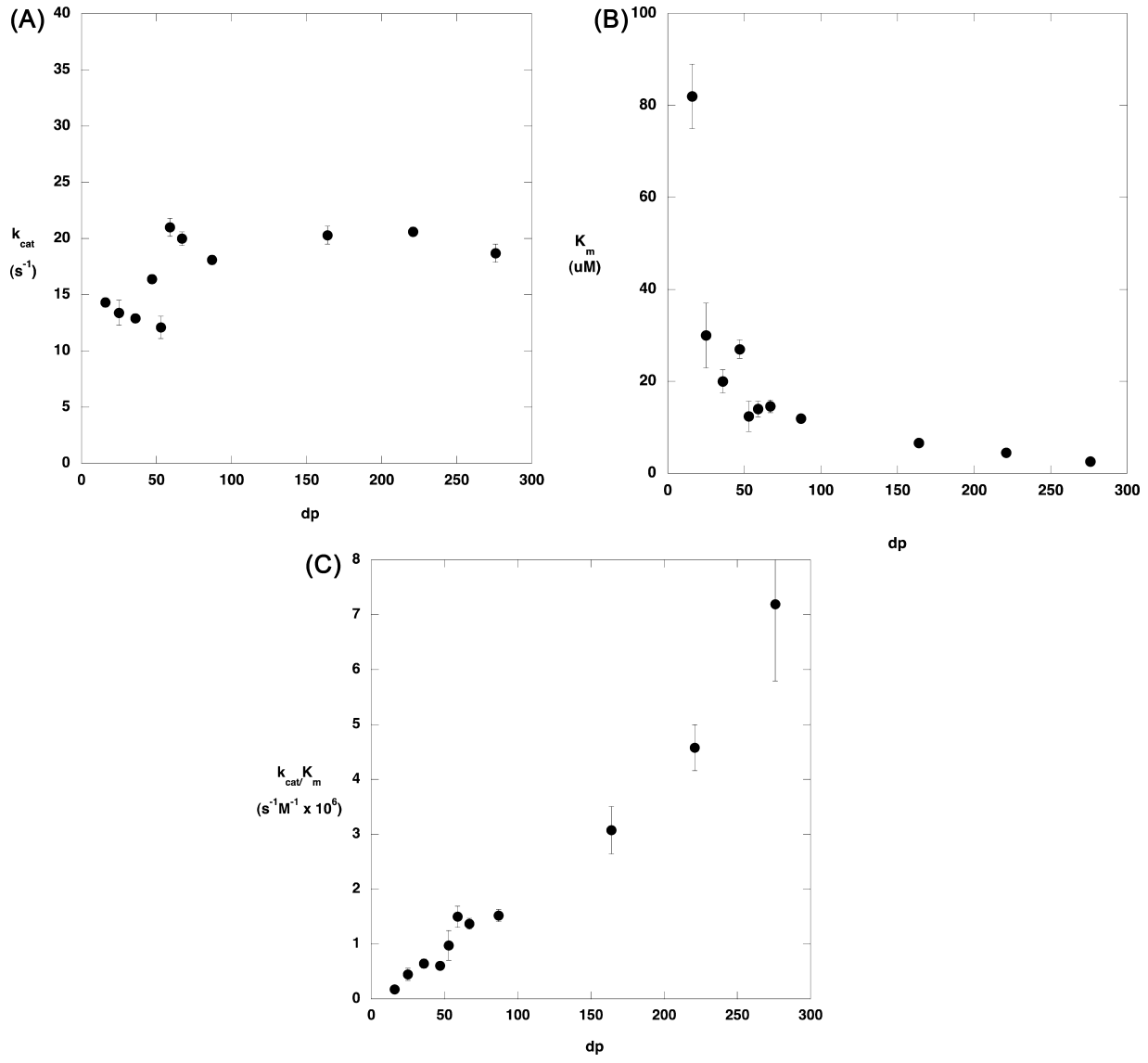


Figure 2. Steady-state kinetic parameters for AlgL-catalyzed degradation of polyM as a function of substrate length: (A) k_{cat}, (B) K_m, and (C) k_{cat}/K_m.

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nonreducing end.[16] The distribution of dimers and trimers was characterized by HPLC analysis and was found to vary with the guluronate content of the sample (Table 2).

Table 2. Composition of AlgL Digestion Products

strain	% guluronate content (starting material)	% dimer (product 1)	% trimer (product 2)
PDO486	40	93	7
PDO300	20	58	42
FRD462	0	39	61

The site of cleavage was identified by ^{1}H NMR spectroscopic characterization of isolated dimeric and trimeric products. Although the same 4,5-unsaturated residue is formed at the nonreducing end of the polymer by the action of lyase at mannuronate or guluronate, the site of action of AlgL can be inferred by comparing the mannuronate and guluronate content of the final product mixture with that of the substrate. The alginate composition was determined by ^{1}H NMR as described previously.[17] The anomeric proton from guluronate residues appears in an uncrowded region of the spectrum, at 5.07 ppm. Overlapping peaks centered at 4.72 ppm arise from H5 on guluronate residues and H1 on mannuronate residues. Multiple peaks for each proton are observed because the chemical shift is dependent on the chemical identity of neighboring residues. H5 protons on guluronate residues that are the central residues in GGM, GGG, and MGG triplets appear away from the 4.72 ppm signal, but GG diads are not found in significant concentrations in alginate isolated from *P. aeruginosa* cultures. Therefore, the peak at 4.72 ppm in the present samples is composed of one proton from guluronate and one proton from mannuronate, so the ratio of the areas of the peaks at 5.07 and 4.72 ppm gives the fractional guluronate content of the alginate. As shown in Figure 3, the guluronate content of the alginate from *P. aeruginosa* strain PDO486 was 40%. The chemical shift of H4 in the unsaturated residue at the nonreducing end of the product is sensitive to the identity of its neighboring residue, so that the peak at 5.95 ppm arises from terminal residues adjacent to guluronate and the peak at 5.86 ppm arises from terminal residues adjacent to mannuronate. On the basis of the areas of these signals, 23% of the residues adjacent to the terminal residue are guluronates; because the guluronate content of the product decreased relative to that of the starting material, AlgL must be converting some guluronate residues into the 4,5-unsaturated residue at the nonreducing end of the product, i.e., AlgL acts on guluronate residues as well as mannuronate residues.

Site of Action. To determine whether AlgL acted at internal sites in its polysaccharide substrate or at the end, the formation of product from a long substrate was monitored in the pre-steady-state time regime (Figure 4). If AlgL catalyzed cleavage at internal sites of the substrate, then several rounds of catalysis would be required before dimeric and trimeric product would be formed. Conversely, if AlgL acts at the ends of the substrate, each catalytic cycle would release dimeric or trimeric product. With a turnover number of approximately 30 s^{-1}, the first catalytic cycle should be completed in ~0.03 s. The time course for product formation extending through the first several cycles revealed a burst of trimer formation, indicating that AlgL acts at the end of its polysaccharide substrate. Dimers were also observed, but at much lower concentrations.

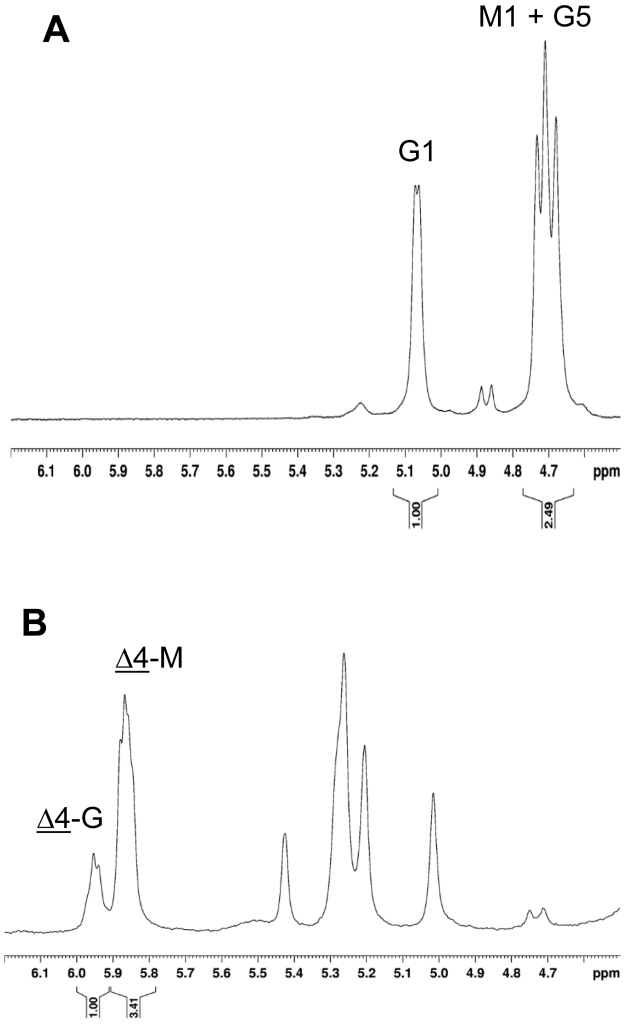

Figure 3. ^{1}H NMR spectra of PDO486 alginate before (A) and after (B) digestion with AlgL. G and M refer to guluronate and mannuronate, respectively; the number is the proton from which the signal arises. Δ-G and Δ-M refer to unsaturated residues at the nonreducing end of the products adjacent to guluronate and mannuronate, respectively. The area under each labeled peak is given below the chemical shift axis.

DISCUSSION

The presence of a gene encoding an enzyme that degrades alginate in the operon for alginate synthesis seems paradoxical, although it is not unusual. The biosynthetic operons for other secreted polysaccharides produced by *P. aeruginosa* also contain genes for a lyase or hydrolase.[6] Nevertheless, assignment of functions to these enzymes remains speculative. Genetic studies have demonstrated that AlgL is required by *P. aeruginosa*; in its absence, alginate or a precursor accumulates in the periplasm leading to rupture of the cells.[7] To gain further insight into the role of AlgL in synthesis and secretion of alginate, we have examined its substrate specificity. In the alginate biosynthetic pathway, the first polymeric product is mannuronan, a homopolymer of β-D-mannuronate residues (polyM). AlgG, a periplasmic enzyme, converts some mannuronate residues into α-L-guluronate residues through epimerization at the C5 position. Mature alginate is acetylated at O2 and O3 on some mannuronate residues, but the sequence in which epimerization and acetylation occur has not been established; therefore, any of the polysaccharides that were examined in this

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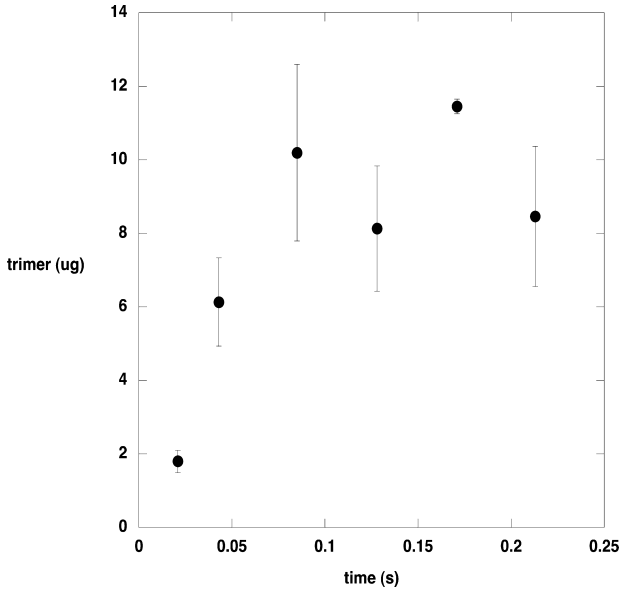


Figure 4. Pre-steady-state product formation by AlgL. Products were separated from the quenched reaction mixture by HPLC and quantitated as described in the text.

study are potential natural substrates for AlgL. If the primary role of AlgL were to degrade extracellular alginate, then the enzyme would be expected to show a preference for acetylated alginate. In fact, the opposite is observed; k_{cat}/K_m values for unacetylated polyM and alginate are approximately 10-fold higher than the values for the acetylated polysaccharides. Because the unacetylated polysaccharides are not found outside the periplasmic space, it seems likely that the primary substrates for AlgL are alginate or polyM that are within the periplasmic space. A Δ*algL* mutant strain with an inducible *alg* operon was constructed by Ohman to investigate the role of AlgL.[7] Following induction, the bacteria were lysed after several hours, and electron micrographs suggested that a polymer accumulated in the periplasm until cell lysis occurred. These observations formed the basis for the proposal that AlgL is a component of a multiprotein scaffold that serves to shepherd alginate through the periplasm to AlgE, a porin-like protein through which alginate is secreted. The kinetic data reported here are consistent with the suggestion that the function of AlgL is to degrade mislocalized alginate or polyM.[7] The difference in the kinetic parameters for polyM versus those for alginate are not significant, indicating that AlgL can act with equal facility on the polysaccharide before and after epimerization has occurred.

The variation in the kinetic parameters for the AlgL reaction as a function of substrate length suggests that the enzyme is optimized for acting on polymeric substrates. Although k_{cat} varies only slightly with polymer length, k_{cat}/K_m increases with increasing polymer length and K_m decreases. The relationship between k_{cat}/K_m and polymer length is particularly striking; k_{cat}/K_m increases linearly with no indication that a plateau value is being approached. A consequence of this behavior is that K_m decreases for longer and longer substrates.

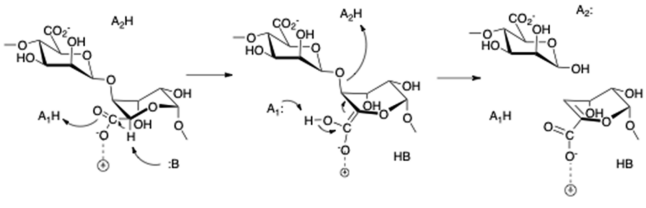
The cause of this phenomenon is interesting to consider. Although K_m is routinely considered as a surrogate measure of substrate affinity, it makes little sense to consider these results in that light. A linear polymer of alginate or polyM longer than 10 residues exceeds the dimensions of AlgL, and although alginate and polyM adopt somewhat structured conformations in solution, it is still difficult to envision how binding interactions between the substrate and enzyme could be distinct for a 150-residue polymer versus a 250-residue polymer. However, the observed kinetic data can be interpreted by considering the meaning of k_{cat}/K_m. Northrop has provided a particularly compelling explanation for k_{cat}/K_m, pointing out that all the microscopic rate constants in the mechanism appear as ratios except for k_1, the bimolecular rate constant for association of the substrate with the enzyme.[18] As a consequence, k_{cat}/K_m is the product of k_1 and a complex ratio that varies between 0 and 1. Thus, k_{cat}/K_m is seen to be a measure of the frequency with which the substrate is productively captured by the enzyme, where productive capture refers to binding events that lead to product formation. The ratio of rate constants that moderates k_1 has a maximal value of 1 in the case that binding of one substrate molecule gives rise to one product. However, if a single substrate binding event can lead to formation of more than 1 equiv of product, k_{cat}/K_m can increase in proportion to the number of equivalents of product formed per substrate binding event. In the case of AlgL, a ready explanation for the increase in k_{cat}/K_m with an increasing substrate length is provided if the enzyme behaves in a processive manner. For an enzyme in which the processivity, that is the number of turnovers that occur before the enzyme dissociates from the substrate, is limited by the length of the polymeric substrate, k_{cat}/K_m should increase with the length of the substrate, because each substrate capture event leads to formation of multiple equivalents of product in proportion to the number of monomeric units in the polymer. The behavior of k_{cat} as a function of substrate length depends on the relative rates of substrate binding and product release. The case in which k_{cat} reaches a plateau value, as was observed with AlgL, occurs when product release is rapid. When substrate is limiting (k_{cat}/K_m conditions), the rate of the reaction is limited by binding of the substrate to the enzyme; longer substrates generate more product once they are bound, so k_{cat}/K_m varies directly with substrate length. When the substrate is saturating, binding is not rate-limiting and the amount of product formed per unit time is independent of whether the product comes from a single long substrate or multiple equivalents of shorter substrate, and the result is that k_{cat} does not vary with substrate length.

Exhaustive digestion of alginate or polyM with AlgL leads to a mixture of dimeric and trimeric products, which we have characterized by ^{1}H NMR spectroscopy and mass spectrometry. To determine whether the products arise from sequential removal of dimers and trimers from the end of the polysaccharide substrate or from repeated cleavage at internal sites until dimers and trimers are formed, we examined the time course for product formation in the presteady state. If AlgL cleaved at internal sites, several catalytic cycles would have to occur before small products appeared and the time course would exhibit a lag in the appearance of dimers and trimers. On the other hand, removal of dimers and trimers from the end of the substrate would yield a time course in which dimers and trimers were immediately apparent. The results of rapid-mixing chemical quench studies of AlgL-catalyzed turnover of alginate are shown in Figure 4. The alginate used in this experiment was converted to mostly trimers by AlgL, and it is clear that there is no lag in trimer production. These data argue that AlgL acts at the end of the alginate molecule, removing successive dimers and trimers as the reaction proceeds.

Because AlgL acts with equal facility on alginate and polyM, we sought to determine whether it cleaves between mannuronate residues exclusively or between mannuronate and guluronate residues. Early work on AlgL established that it did not cleave between adjacent guluronate residues[8] but did not address whether it could catalyze scission of mannuronate-(β1→4)guluronate or guluronate(α1→4) mannuronate linkages. Because the reaction is a β-elimination arising from abstraction of the proton at C5 on the residue closer to the reducing end and mannuronate and guluronate are C5 epimers, the same unsaturated residue is formed from the action of the enzyme at mannuronate and guluronate residues. However, the residues from which protons were abstracted can be inferred by comparison of the relative proportions of mannuronate and guluronate in the starting polysaccharide and in the products. The protocol for determining the composition of alginate using ^{1}H NMR spectroscopy is well-established.[19] The composition of the dimeric and trimeric products can be determined by ^{1}H NMR spectroscopy as well, because the chemical shift of H4 on the unsaturated terminal residue is sensitive to whether the residue is adjacent to mannuronate or guluronate.[17] It is clear that AlgL can cleave between adjacent mannuronate residues, because polyM is a substrate. If AlgL cleaved mannuronate- and guluronate-containing alginate by abstracting mannuronate protons exclusively, no guluronate residues would be converted to unsaturated residues, and the fractional guluronate composition of the products would increase. However, as shown in Figure 3, the guluronate content in the products was lower than in the starting material, implying that cleavage occurred at guluronate residues as well as mannuronates.

The finding that AlgL catalyzes a *syn* elimination, by abstraction of a proton from a mannuronate residue, and an *anti* elimination, by abstraction of a proton from a guluronate residue, is not unprecedented for a polysaccharide lyase[20] but is unusual from a mechanistic point of view. In addition to the fact that absolute stereochemical specificity is the norm in enzyme-catalyzed reactions, it has been suggested that the very high pK of the proton adjacent to the carboxylate in the substrate would lead to a mechanism requiring a general base and two general acids, and *anti* elimination is expected to prevent futile proton transfer between the general base and general acid.[21] Gerlt and co-workers have proposed a general chemical mechanism for β-elimination reactions that addresses the thermodynamic difficulties inherent in abstraction of a proton α to a carboxylate; transposed onto the AlgL reaction, this mechanism consists of concerted neutralization of the carboxylate and general base-catalyzed abstraction of the C5 proton, followed by elimination of the substituent at C4 (Scheme 2). Alginate lyase from *Sphingomonas* sp. A1 catalyzes the *syn* elimination required for cleavage between adjacent mannuronate residues; structural studies revealed a critical tyrosine residue at the active site, Y246, that was proposed to serve as both a general base and a general acid in a reaction in which the proton is abstracted from C5 and transferred to the leaving group in a single step.[22] The active site residues are conserved between the *Sphingomonas* enzyme and AlgL, and mutation of the homologous Y256 residue in AlgL does cause a substantial decrease in activity (E. Farrell, unpublished results). Whether Y256 is the only residue involved in proton transfers in AlgL-catalyzed cleavage at mannuronate residues remains to be established; the *anti* elimination that occurs during cleavage between mannuronate and guluronate cannot be mediated by a single residue. Two scenarios can be envisioned by which AlgL could catalyze both *syn* and *anti* eliminations. In the first, the enzyme would have two different general bases, one for each epimeric substrate. Alternatively, two binding modes would be available, so that the same general base could act upon each epimer. Chondroitin lyase ABC catalyzes cleavage of chondroitin sulfate and dermatan sulfate, which requires abstraction of the C5 proton from the epimers glucuronate and iduronate. It appears that the enzyme has two overlapping active sites, with residues at one site required for cleavage of both substrates, but a pair of required histidine residues at the second site that are required only for cleavage of dermatan sulfate.[23] Heparinase II has an active site with the same constellation of amino acids that are found in the AlgL active site, and it also is capable of catalyzing *syn* and *anti* β-eliminations. It has been proposed that the activity results from the substrates binding in different orientations at the same active site.[24] Mutagenesis studies have identified some critical active site residues, but the identities of the general acid and general base have not been determined with certainty. Mechanistic and structural studies of AlgL are underway to explore further details of acid−base catalysis in alginate cleavage.

Scheme 2



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ABBREVIATIONS

DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; polyM, homopolymer of mannuronate residues, also called mannuronan; Tris, tris(hydroxymethyl)aminomethane base; MES, 2-(*N*-morpholino)ethanesulfonic acid; dp, degree of polymerization.

ADDITIONAL NOTE

[a]Alginate isolated from seaweed is widely used in a variety of industrial and pharmaceutical applications and is not acetylated. *Pseudomonas* alginate is acetylated; however, for the sake of clarity in this work, we will use the term alginate to refer to deacetylated material and acetylated alginate to refer to material that has not been chemically deacetylated.

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