The Recombinant *Azotobacter vinelandii* Mannuronan C-5-Epimerase AlgE4 Epimerizes Alginate by a Nonrandom Attack Mechanism*

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The Ca\(^{2+}\)-dependent mannuronan C-5-epimerase AlgE4 is a representative of a family of *Azotobacter vinelandii* enzymes catalyzing the polymer level epimerization of \(\beta\)-D-mannuronic acid (M) to \(\alpha\)-L-guluronic acid (G) in the commercially important polysaccharide alginate. The reaction product of recombinantly produced AlgE4 is predominantly characterized by an alternating sequence distribution of the M and G residues (MG blocks). AlgE4 was purified after intracellular overexpression in *Escherichia coli*, and the activity was shown to be optimal at pH values between 6.5 and 7.0, in the presence of 1–3 mM Ca\(^{2+}\), and at temperatures near 37 °C. Sr\(^{2+}\) was found to substitute reasonably well for Ca\(^{2+}\) in activation, whereas Zn\(^{2+}\) strongly inhibited the activity. During epimerization of alginate, the fraction of GMG blocks increased linearly as a function of the total fraction of G residues and comparably much faster than that of MMG blocks. These experimental data could not be accounted for by a random attack mechanism, suggesting that the enzyme either slides along the alginate chain during catalysis or recognizes a pre-existing G residue as a preferred substrate in its consecutive attacks.

Alginites are linear copolymers of \(\beta\)-D-mannuronic acid (M)\(^1\) and its C-5-epimer, \(\alpha\)-L-guluronic acid (G). The relative amounts as well as the distribution of these two residues vary greatly between alginites from different species of both brown alga (1–4) and bacteria (5, 6). Biologically, alginate appears to serve a variety of functions. Brown algae maintain rigidity and flexibility through alginites of various sequential structures constituting different tissues of the plants (1). In *Azotobacter vinelandii*, alginites are major constituents of the vegetative capsule (7) and of the rigid and dessication-resistant walls of metabolically dormant cysts. This resting stage may be entered in nitrogen-free medium containing certain carbon sources, such as \(\beta\)-hydroxybutyrate (8). Alginate synthesized by the opportunistic pathogen *Pseudomonas aeruginosa* appears to have multiple roles in pathogenesis: as an immunomodulator (9), as a virulence factor by protecting the bacterium against phagocytosis (10), and in the formation of biofilms that contribute to antibiotic resistance (11) and stable attachment to cell surfaces (12). A role for alginate in the phytopathogenesis of certain pseudomonades has also been proposed (13).

The parameters that form the basis for the range of properties and hence the commercial value of alginites are the chain length and the fraction and sequential distribution of M and G residues in the polymer. An alginate rich in G blocks (stretches of consecutive G residues) converts into a gel by cross-linking with divalent ions. Such gelling properties are highly appreciated in the food, pharmaceutical, dental, textile, and paper industries (14) and render this type of alginate a good material for immobilization of biocatalysts (15, 16). Alginic acid gels, formed by lowering of pH, may broaden the application range of alginites at conditions that require such conditions, e.g. in the development of drug delivery systems (17, 18). In this context, alginites with a preponderance of MG blocks (stretches of alternating M and G residues) may be a valuable resource due to the high acid solubilities displayed by this type of polymer (3). Alginites with a high mannuronic acid content have acquired considerable biomedical interest due to its antitumor (19) and cytokine production stimulatory (20) activities.

Mannuronan C-5-epimerase was first discovered in a culture medium of *A. vinelandii* (21) and was found to catalyze the Ca\(^{2+}\)-dependent epimerization of M into G at the polymer level (22). This reaction represented the first example of an epimerase acting on a polymeric substrate (23). In the early investigations, the enzyme was reported to introduce single G residues or homopolymeric blocks of G, depending on the concentration of calcium ions in the growth medium (22) or in the incubation mixture (24). Screening of an *A. vinelandii* gene library has, however, proved the existence of a family of seven mannuronan C-5-epimerase genes, *algE1*, *algE2*, *algE3*, *algE4*, and *algE5* (25) and *algE6* and *algE7* (53) six of which are clustered in the genome. The activities of the protein products of these genes are Ca\(^{2+}\)-dependent, and they contain repeats of two types of structural units, designated A and R. The 150-amino acid R module is present in one (*AlgE4*) to seven (*AlgE3*) copies. Each R module furthermore contains four to six repeating sequences corresponding to putative Ca\(^{2+}\)-binding motifs. The function of the 385-amino acid A module, present in one or two copies, is unknown at present (25, 26). Both modules are highly conserved at the amino acid sequence level among the members of the protein family, indicating duplication and rearrangement events during evolution (25). The variability in the number of A and R repeats, the deduced molecular masses of the AlgE epimerases vary in the range of 57.7–191 kDa. Also encoded in the *A. vinelandii* genome is a Ca\(^{2+}\)-independent mannuronan C-5-epimerase, AlgG (27), which

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\(^{1}\) The abbreviations used are: M, \(\beta\)-D-mannuronic acid; G, \(\alpha\)-L-guluronic acid; MOPS, 3-(N-morpholino)propanesulfonic acid; MES, 2-(N-morpholino)ethanesulfonic acid; PIPES, piperezine-N,N'-bis(2-ethanesulfonic acid); CAPS, 3-(cyclohexylamino)propanesulfonic acid.

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does not share any significant overall amino acid sequence homology with AlgE1–5 and whose epimerization product is yet unknown.

Based on the enzymes studied so far (AlgE2 and AlgE4), it is clear that each epimerase catalyzes the production of alginites with distinct monomer distribution patterns (25, 26). In this paper, a more detailed study of the mechanistic and biochemical properties of AlgE4 is reported. This enzyme predominately produces an alginate with long stretches of alternating MG sequences. The kinetics of formation of this sequence pattern could not be accounted for by a random attack model and therefore led us to suggest alternative modes of action of the epimerase.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids**

pHH4 (25), a previously published derivative of the commercially available expression vector pTrc99A, was used to express algE4 in the Escherichia coli strain JM1105 (28).

**Expression and Preparation of a Crude AlgE4 Fraction to Be Used for Purification**

A typical purification scheme involved 4 liters of 3× concentrated LB medium (29), supplemented with 0.5 mg/ml ampicillin. One-liter cultures were inoculated to 1% from an overnight culture of JM105(pHH4) in the same medium. After 2.5 h of incubation at 37 °C with shaking, AlgE4 was eluted with isopropyl-β-thiogalactopyranoside at a concentration of 0.25 mM. AlgE4 production was allowed for a maximum of 3 h following induction. The cells were then harvested, resuspended in 1/10 volume of 10 mM MOPS, pH 6.8, and ultrasonicated. The broken cells were centrifuged at 16,300 × g for 30 min, and the resulting supernatant was filtered through a sterile Milllex-GV membrane (0.22-μm pore size, Millipore) prior to activity measurements and further purification. This crude extract was designated Fraction A.

**Purification of AlgE4**

Ion Exchange Chromatography—Fraction A was loaded (100 ml/run) onto a HiLoad 210/10 Q-Sepharose HP column (Pharmacia) equilibrated with 10 mM MOPS, pH 6.8. AlgE4 was eluted with a stepwise 0–1 mM NaCl gradient (in 10 mM MOPS, pH 6.8), and the enzyme was eluted between 0.35 and 0.45 mM NaCl. Fractions containing mannnurons C-5 epimerase activity were pooled and designated Fraction B.

Native Polyacrylamide Gel Electrophoresis Using Model 491 Prep Cell (Bio-Rad)—136 mg of protein in an 1 mg/ml solution, corresponding to Fraction B, was concentrated to 11 mg/ml on a 5 ml HiTrap Q-Sepharose HP column (Pharmacia). This solution (10 ml) was dialyzed against Tris/glycine electrode running buffer in a Slide-A-Lyzer dialysis cassette (Pierce) and further concentrated to 36 mg/ml within the slide. To one half (1 ml) of this sample, an equal volume of sample buffer (without glycerol) was added. The sample was then loaded onto the stacking gel surface, and electrophoresis was run at 4 °C at an initial voltage of 250 V and at a final voltage of 400 V. The elution rate was varied within the range of 0.3–1 ml/min. Separating (8%) and stacking gels were 10 and 3.5 cm in height, respectively. Gel, sample, and running buffers were made according to Ornstein (30) and Davis (31) and as described by the manufacturer. Eluted fractions containing AlgE4 activity were pooled and concentrated as before application onto the Prep Cell, except that the dialysis step was performed against 10 mM MOPS, pH 6.8. The resulting, highly purified extract contained 9.6 mg protein, and 7.5 and 40 mg, respectively. All four diad combinations, i.e. F_{MGO}, F_{G3M}, F_{G3M}, and F_{GGO}, and triad sequences were determined for various extents of conversion. Substrates with an M content of about 96% were obtained from P. aeruginosa (5). The alginites were deacetylated (5) and partially degraded by mild hydrolysis (33) to a degree of polymerization (DP) of 20–40, prior to epimerization. Except where otherwise stated, epimerization reactions were carried out with 10 μg of enzyme/mg alginate in 10 mM MOPS, pH 6.8, 1.5 mM CaCl_{2}, and in a total volume of 6 ml at 37 °C. Reaction times are described in figure legends. Reactions were terminated by chelation of Ca^{2+} by adding Na_2EDTA (0.5 mM, pH 8.0) to 9 mM. Extensive dialysis of samples against distilled water was performed before preparation for nmr spectroscopy (33).

**Biochemical Characterization of AlgE4**

The effect of pH on the activity of purified AlgE4 was investigated using buffers with buffering capacities covering different pH ranges (34). The buffers used were MES (pH 4.15–5.6), PIPES (pH 6.2–7.3), HEPES (pH 7.5–8.2), and CAPS (pH 9.5–10.5). Each buffer was used at a 10 mM concentration, and the pH values were adjusted with NaOH or HCl. Activity was measured as dpm/μg protein, and characterization results were expressed as percentages of maximum activity. All measurements were performed in duplicate.

**AlgE4 Kinetics**

Apparent kinetic constants were estimated from the direct linear plot of Cornish-Bowden and Eisenthal (35). 0.1 μg of enzyme was incubated for 5–10 min at 37 °C in 10 mM MOPS, pH 6.8; 0.4, 1.5, or 5 mM CaCl_{2}; and 12–150 μM alginate (sugar residues). All assays, including controls without enzyme, were performed in triplicate.

To investigate the reversibility of the enzyme reaction, the unlabeled M-rich P. aeruginosa alginate was used as substrate, as for the nmr studies. Due to the high activity of the enzyme on this substrate, incorporation of tritium from labeled water into M residues (indicating reversibility) could easily be monitored. One might also use a G-rich substrate (epimerized in tritiated water) as a starting material and followed the release of tritium from G residues into unlabeled water. However, the interpretation of such an experiment would be difficult, because the epimerization reaction (M to G), which does not reach a clear end point (see "Results"), would also contribute to tritium release. 20 mg of alginate was epimerized with 200 μg of AlgE4 (10 μg enzyme/mg alginate) for 4 h, in the presence of H$_2$O (total activity of 2.3 × 10^{10} dpm) and under conditions otherwise identical to those used in the nmr studies. Also included in the experiment was a control sample with no enzyme. After precipitation of the alginate as described for the radioisotope assay procedure, the samples were completely hydrolyzed and prepared for ion exchange chromatography (36). Separation of sugar monomers was performed with 0.5–2 M acetic acid at a flow rate of 2.4 ml/min (36). The total carbohydrate content in the eluted fractions was determined using the phenol-sulfuric reaction (37). The concentration of uronic acids corresponding to an optical density of 0.1 at 485 nm is 15.5 μg/ml for mannuronic acid and 9.2 μg/ml for guluronic acid (38).

**Protein Quantification and Sequencing**

SDS-polyacrylamide gel electrophoresis, using a 7.5% gel, was performed according to Laemmli (39), and the gel was stained using SYPRO™ Orange Stain (Bio-Rad). Molecular masses were determined relative to mobilities of protein standards.

For N-terminal sequencing analyses, proteins were blotted onto a polyvinylidene difluoride membrane (Immobilon), Coomassie stained, and analyzed on an Applied Biosystems 477A amino acid sequencer. For amino acid composition analysis, a protein sample of known A$_{pro}$ was lyophilized and hydrolyzed to its component amino acids in 6 M HCl at 110 °C for 24 h in vacuo. Quantitative amino acid composition analysis was determined using an Applied Biosystems model 421 automatic
Properties of AlgE4

Amino acid analyzer. The extinction coefficient calculated from these data was used in spectroscopic determinations of AlgE4 concentrations. Alternatively, the AlgE4 concentration was determined according to Bradford (40) using bovine serum albumin as standard. The two methods gave results differing less than 5%.

Mathematical Modelling of the Epimerization Mechanism

The "random attack" model, described under "Results," was simulated by a Monte Carlo scheme (41) that takes into account the inaccessible M residues flanking G residues. This was done by using an ensemble of alginate chains with a degree of polymerization (DPn) of 30 and simulating the epimerization by randomly selecting residues in the ensemble, disallowing epimerization of previously epimerized residues or their flanking residues. In the "progressive" model, AlgE4 is assumed to randomly attack the mannanuran substrate and epimerize a given residue. Without dissociation of the enzyme-substrate complex, it then epimerizes a new residue by relocating itself two residues along the polysaccharide chain. As a result, a strictly alternating MGMG sequence is introduced. The processivity refers to the average number of converted residues per attack and is given by \( \frac{1}{1 - p} \), where \( p \) is the probability of relocation along the chain and \( (1 - p) \) is the probability of dissociation of the enzyme-substrate complex. In terms of triad fractions it can be shown that for AlgE4, \( P = \left( F_{MMG} - F_{MGGM} \right) \), where \( t \) is the time at which the epimerization reaction is stopped. The model was implemented using a Monte Carlo scheme (41, 42) based on an ensemble of alginate chains with a degree of polymerization (DPn) of 30, as in the random attack model. It was additionally assumed that the enzyme relocates only in one direction along the substrate. Furthermore, the number of enzyme-substrate complexes was continuously updated in accordance with the changing substrate concentration (defined as the concentration of M residues not neighboring G residues). In the "preferred attack" model, the enzyme is assumed to preferentially epimerize residues proximal to M residues that have a neighboring G. To accomplish this, it binds to intrapolymer GMMM sequences with a larger binding constant than to MMMMM segments. Consequently, the fraction of GMGM becomes higher than that of MMGM following epimerization. Because AlgE4 is assumed not to epimerize M residues neighboring G residues, the number of sequences that can act as substrates is limited. The polymer is thus considered to consist of two different types of substrates, GMGM and MMMM, competing for the pool of enzymes. Although the copolymer nature of the substrate indicates that several types of substrates coexist in the alginate chains, this two-substrate approximation is the simplest extension over the one-substrate model that can be realized. The preferred attack model was implemented using a Monte Carlo scheme (43, 44) on the basis of alginate chains with a degree of polymerization (DPn) of 30, as above.

The simulation models are available upon request.

RESULTS

Expression and Purification of AlgE4—The plasmid pH4 was used to overexpress AlgE4 intracellularly in E. coli, and the enzyme was finally purified from this source. The level of expression was relatively low (Fig. 1, lane 2), but following ion exchange chromatography the specific activity increased 6-fold (Table I, Fraction B) relative to that of the crude extract (Fraction A). Interestingly, the four main bands present in Fraction B (Fig. 1, lane 3), corresponding to molecular masses of 79, 74, 68, and 58 kDa, were absent in control cells lacking the algE4 gene. The proteins in these bands were all subjected to N-terminal sequence analysis, and the results showed that the sequences in all four cases were identical to the deduced sequence of AlgE4. Thus, it appears that more than one form of the enzyme is being made in E. coli.

A further 2-fold increase in specific activity was obtained by native polyacrylamide gel electrophoresis (Table I, Fraction C), and the epimerase activity was found to correlate with the band corresponding to a 79-kDa protein (Fig. 1, lane 4). This is a significantly higher molecular mass than to be expected on the basis of the deduced sequence (57.7 kDa). In this purification step the 58-kDa protein was also purified to near homogeneity, and the corresponding fraction was found not to display epimerase activity. The nature of this and other forms different from the 79-kDa protein has not been further studied.

The extinction coefficient for AlgE4, which was used in specific activity measurements, was determined to be 0.89. The concentration of AlgE4 could then be calculated as A280/0.89 mg/ml.

Biochemical Characterization of AlgE4—All characterization experiments were carried out on the basis of Fig. 2a, which shows that AlgE4 activity is linear for long incubation times in the presence of excess substrate. The temperature optimum is close to 37 °C, but the enzyme activity is maintained at a broad range of temperatures. Preincubation of the enzyme affects the activity only at temperatures as high as 50–60 °C (Fig. 2b).

The pH activity curve showed that AlgE4 has an optimum between 6.5 and 7.0 and that the enzyme activity is very sensitive to alkaline pH values. At pH values above 8 virtually all activity was lost (data not shown). The AlgE4 activity was found to be nearly optimal in the absence of NaCl, but additions up to 0.1 M did not reduce the activity much. As the concentration increased beyond this value, activity dropped, and at 0.5 M NaCl less than 10% of the activity was retained (data not shown).

Because the activities of the epimerases have long been known to depend on Ca2+, this parameter was analyzed in more detail (Fig. 2c). Optimal AlgE4 activity was obtained at around 1–3 mM Ca2+ at a substrate concentration (sugar residues) of 0.5 mM or higher. At lower concentrations of substrate, the optimal Ca2+ concentration was somewhat reduced. The

![Fig. 1. SDS-polyacrylamide gel electrophoresis of the purification of recombinant AlgE4. Lane 1, standards; lane 2, crude extract (16 μg applied); lane 3, following Q-Sepharose HP ion exchange chromatography (3 μg applied); lane 4, following native polyacrylamide gel electrophoresis (1 μg applied).](image)

**Table I**

| Purification of mannanuran C-5-epimerase AlgE4 |
|-----------------------------------------------|
| Total protein (mg) | Total enzyme activity (dpm 3H/min × 10⁻³) | Specific activity (dpm 3H/min/mg protein × 10⁻³) | Recovery of enzyme activity (%) | Purification fold |
|-------------------|-----------------------------------------|--------------------------------------------|-------------------------------|------------------|
| Crude extract (Fraction A) | 988 | 73 | 7.4 | 100 | 1 |
| HiLoad Q-Sepharose HP (Fraction B) | 136 | 57 | 42 | 78 | 5.7 |
| Native polyacrylamide gel electrophoresis (Fraction C) | 30 | 24 | 87 | 33 | 12 |
negative effects of adding more Ca\(^{2+}\) were moderate. Experiments were carried out to assure that no substrate inhibition was occurring, to an upper limit of 1.5 mM. Chelation of Ca\(^{2+}\) with Na\(_2\)EDTA at equimolar concentrations (1 mM) resulted in a complete inhibition of activity, whereas a reduction of the Na\(_2\)EDTA concentration to half (0.5 mM) had a much less pronounced effect. Interestingly, the epimerase activity of AlgE4 preincubated with 1 mM Na\(_2\)EDTA could be restored by the addition of a molar excess of Ca\(^{2+}\) (data not shown).

The effects of divalent cations other than Ca\(^{2+}\) on an A. vinelandii mannosan C-5-epimerase have been investigated previously (24). The exact identity of this enzyme is, however, unknown. The data in Fig. 2d demonstrate that Sr\(^{2+}\) could substitute for Ca\(^{2+}\) with an efficiency of about 30%. In contrast, Mg\(^{2+}\), Mn\(^{2+}\), Ba\(^{2+}\), and Zn\(^{2+}\) had no stimulatory effect on the epimerization reaction in the absence of Ca\(^{2+}\). At suboptimal concentrations of Ca\(^{2+}\) (0.4 mM), the addition of an equimolar amount of Sr\(^{2+}\), Mg\(^{2+}\) or Mn\(^{2+}\) stimulated the activity, whereas Ba\(^{2+}\) and Zn\(^{2+}\) had the opposite effect (data not shown). At optimal Ca\(^{2+}\) concentrations, on the other hand, all ions tested (except Mg\(^{2+}\)) were inhibitory (Fig. 2d). The inhibitory effect was particularly strong with Zn\(^{2+}\).

Specificity of the Epimerization Reaction—In the early studies of the mannosan C-5-epimerase reaction, it was observed that an enzyme fraction from A. vinelandii gave rise to different epimerization patterns in the alginate, dependent on the Ca\(^{2+}\) concentration used during incubation (22). A possible interpretation of this is that the G distribution pattern in the reaction product of a given enzyme depends on the Ca\(^{2+}\) concentration or that more than one enzyme was present in the A. vinelandii fraction. Each enzyme might then be affected differently by the variations in the Ca\(^{2+}\) concentration (45). Fig. 3a shows that the AlgE4-catalyzed reaction led to the formation of almost exclusively MG blocks under all Ca\(^{2+}\) concentrations tested. It therefore appeared that the enzyme is very specific with respect to the sequence distribution of G residues in its reaction product, and the lack of homopolymeric G blocks indicates that the alginate formed by AlgE4 is of a non-gel-forming type (46).

During the studies described above we sometimes noticed that under certain nonstandard conditions a small but significant amount of GGG and MGG triads were introduced in the alginate as a result of the AlgE4 activity. To investigate this further we incubated the substrate in the presence of a large excess of enzyme and analyzed the epimerization pattern by nmr as a function of time. Under these conditions, a significant amount of GGG and MGG triads were introduced in the reaction product of a given enzyme. This is best demonstrated that Sr\(^{2+}\) permitted the AlgE4-catalyzed reaction led to the formation of almost exclusively MG blocks under all Ca\(^{2+}\) concentrations tested. It therefore appeared that the enzyme is very specific with respect to the sequence distribution of G residues in its reaction product, and the lack of homopolymeric G blocks indicates that the alginate formed by AlgE4 is of a non-gel-forming type (46).

Kinetikis of Epimerization by AlgE4—The determination of kinetic constants for an enzyme such as AlgE4 is complicated by the fact that the substrate changes throughout the reaction. Classical Michaelis-Menten kinetics therefore does not apply. However, the reaction proceeded linearly at a wide range of substrate concentrations. Furthermore, no substrate inhibition was observed at very high concentrations. The \(V_{\text{max}}\) and \(k_{\text{cat}}\) values could therefore be determined and were found to be 14.8 \(\mu\)mol min\(^{-1}\) mg\(^{-1}\) protein and 14 s\(^{-1}\), respectively. For determination of the apparent \(K_m\) value, the specific activity of the substrate represents a technical limitation. The labeled substrate was prepared by growing cells on tritiated glucose, which limits the possibilities of preparing substrates with specific activities high enough to assay enzyme activity at the generally preferred lower concentration limit of 0.2 \(K_m\). The Cornish-Bowden-Eisenthal direct line plot analysis was nevertheless performed down to the manageable lower limit of 0.67 \(K_m\). This yielded a \(V_{\text{max}}\) value of 14 \(\mu\)mol min\(^{-1}\) mg\(^{-1}\) protein, which is in good agreement with that obtained by simply assaying activity at high substrate concentrations.

An apparent \(K_m\) value
The Mechanism of the Epimerization Process—The fact that AlgE4 introduces an alternating sequence of M and G residues into its substrate raises the question of how such a pattern is created. One possibility is that the enzyme after each epimerization event leaves its substrate and then randomly selects a new M residue from any of the polymer molecules in the solution (random attack model) (49). An important exception to this random pattern of attack would be that M residues flanking pre-existing G residues would not be selected. Experimentally, we used nmr spectroscopy to evaluate this model by following the fractional distribution of sugar sequence triads in the reaction product as more and more G residues were being introduced by the activity of the enzyme (Fig. 5). The peaks in the resulting spectra were integrated, and the triads thus determined were plotted as a function of total G content (FG) (Fig. 6). We therefore conclude that the random attack model does not adequately describe the course of the epimerization process. Instead we propose two other models; the preferential attack model and the processive attack model.

Properties of AlgE4

The properties of AlgE4 were studied by measuring the concentration of G and M monomers, respectively (38).

The first and second peaks correspond to 4 mM Ca, 1 mM Ca, and 0.1 mM Ca, respectively. The third peak corresponds to 0.05 mM Ca, and the fourth peak corresponds to 0 mM Ca.

A proposed mechanism of epimerization of a uronate residue involves three steps: the neutralization of the negative charge, the abstraction of the proton at C5, and finally, the replacement of the abstracted proton by a proton donor and a concomitant flipping of the C4-configured G residues (47). Water is shown to function as such a donor (23, 48), and on this basis we investigated the degree of reversibility of the epimerization reaction by incubating non-radioactive alginate with AlgE4 in the presence of tritiated water. By hydrolyzing the epimerized polymer, followed by separation of the two types of uronic acids, the radioisotope could then be specifically located. The results in Fig. 4 show that the activity of the G residues was seven times that of the M residues, indicating that the reaction predominantly proceeds in the M to G direction. Moreover, the labeled M residues might not necessarily originate from reversal of the reaction. Due to the proposed mechanism of epimerization (47), it seems possible that the enzyme to some extent exchanges protons without completing the epimerization reaction.
Purification of AlgE4 to homogeneity by conventional techniques was complicated by the existence of apparently differently sized AlgE4 molecules in the sample following ion exchange chromatography. Proteolysis to various degrees may have occurred at the crude extract level. Proteolytic removal of essential amino acid sequences, with or without a concomitant change in conformation of the truncated molecules, could explain the lack of mannuronan C-5-epimerase activity displayed by at least one AlgE4 form. An alternative and perhaps more probable explanation is that the differences in migration rates (under both denaturing and nondenaturing conditions) are based solely on conformational heterogeneity. AlgE4 (at least recombinant from E. coli) may exist as a pool of conformationally heterogeneous molecules, not all of which are active. Multiple bands in a denaturing gel could then be due to various degrees of denaturation and nondenaturing conditions) are based solely on conformational heterogeneity. AlgE4 (at least recombinant from E. coli) may exist as a pool of conformationally heterogeneous molecules, not all of which are active. Multiple bands in a denaturing gel could then be due to various degrees of denaturation and nondenaturing conditions. Suboptimal binding of SDS may further be reflected in the abnormally high apparent molecular masses of all but the 58-kDa AlgE4 variant. This protein may have been completely denatured and hence has bound SDS unrestrainedly. A further indication of conformational heterogeneity of AlgE4 is the resolution of the AlgE4 pool by hydrophobic interaction chromatography, which we observed in a recent experiment (data not shown).

The ability of Sr$^{2+}$ to substitute for Ca$^{2+}$ in stimulation of epimerase activity may be ascribed to the fact that these ions have similar ionic radii (51). The slightly stimulatory effect of Sr$^{2+}$ in the absence of Ca$^{2+}$, is thus not very surprising. The inhibitory action of Sr$^{2+}$, Mn$^{2+}$, Ba$^{2+}$, and Zn$^{2+}$ at optimal Ca$^{2+}$ concentrations may likewise be explained in terms of competitive binding of enzyme or of substrate by these cations. Alginate has a particularly strong affinity for Ba$^{2+}$ (52), possibly explaining the relatively strong inhibitory action of this cation. The negligible effect of Mg$^{2+}$ at optimal concentrations of Ca$^{2+}$ could likewise be due to the very low affinity of alginate for this type of ion (52). The detrimental effect of Zn$^{2+}$ has also been observed for the Ca$^{2+}$-independent A. vinelandii mannuronan C-5-epimerase AlgG (27), possibly indicating an alternative function of this ion on the action of mannuronan C-5-epimerases in general.

The sequence distribution pattern produced by AlgE4 is mainly alternating with respect to M and G residues. GG blocks may, however, be introduced as a consequence of elevated Ca$^{2+}$ levels in combination with excessive enzyme concentrations and prolonged incubation times. This shows that the enzyme is not totally specific under all conditions.

The analysis of the mode of action of the AlgE4 epimerase clearly abolished the random attack model. Actually, the end product of such an attack would not be a strictly alternating structure, because random attack eventually would yield a substantial fraction of MGMGMGM sequences, which would remain inaccessible to further epimerization. We therefore propose that the enzyme either slides along the alginate chain during the epimerization process (processive model) (49) or

**DISCUSSION**

Purification of AlgE4 to homogeneity by conventional techniques was complicated by the existence of apparently differently sized AlgE4 molecules in the sample following ion exchange chromatography. Proteolysis to various degrees may have occurred at the crude extract level. Proteolytic removal of essential amino acid sequences, with or without a concomitant change in conformation of the truncated molecules, could explain the lack of mannuronan C-5-epimerase activity displayed by at least one AlgE4 form. An alternative and perhaps more probable explanation is that the differences in migration rates (under both denaturing and nondenaturing conditions) are based solely on conformational heterogeneity. AlgE4 (at least recombinant from E. coli) may exist as a pool of conformationally heterogeneous molecules, not all of which are active. Multiple bands in a denaturing gel could then be due to various degrees of denaturation and nondenaturing conditions). Suboptimal binding of SDS may further be reflected in the abnormally high apparent molecular masses of all but the 58-kDa AlgE4 variant. This protein may have been completely denatured and hence has bound SDS unrestrainedly. A further indication of conformational heterogeneity of AlgE4 is the resolution of the AlgE4 pool by hydrophobic interaction chromatography, which we observed in a recent experiment (data not shown).

The ability of Sr$^{2+}$ to substitute for Ca$^{2+}$ in stimulation of epimerase activity may be ascribed to the fact that these ions have similar ionic radii (51). The slightly stimulatory effect of Sr$^{2+}$ in the absence of Ca$^{2+}$, is thus not very surprising. The inhibitory action of Sr$^{2+}$, Mn$^{2+}$, Ba$^{2+}$, and Zn$^{2+}$ at optimal Ca$^{2+}$ concentrations may likewise be explained in terms of competitive binding of enzyme or of substrate by these cations. Alginate has a particularly strong affinity for Ba$^{2+}$ (52), possibly explaining the relatively strong inhibitory action of this cation. The negligible effect of Mg$^{2+}$ at optimal concentrations of Ca$^{2+}$ could likewise be due to the very low affinity of alginate for this type of ion (52). The detrimental effect of Zn$^{2+}$ has also been observed for the Ca$^{2+}$-independent A. vinelandii mannuronan C-5-epimerase AlgG (27), possibly indicating an alternative function of this ion on the action of mannuronan C-5-epimerases in general.

The sequence distribution pattern produced by AlgE4 is mainly alternating with respect to M and G residues. GG blocks may, however, be introduced as a consequence of elevated Ca$^{2+}$ levels in combination with excessive enzyme concentrations and prolonged incubation times. This shows that the enzyme is not totally specific under all conditions.

The analysis of the mode of action of the AlgE4 epimerase clearly abolished the random attack model. Actually, the end product of such an attack would not be a strictly alternating structure, because random attack eventually would yield a substantial fraction of MGMGMGM sequences, which would remain inaccessible to further epimerization. We therefore propose that the enzyme either slides along the alginate chain during the epimerization process (processive model) (49) or

**Fig. 5.** Sequence distribution pattern produced by AlgE4, monitored by $^{13}$C nmr spectroscopy of epimerized alginate. Reactions were incubated for 0 ($F_{MM}=0.04$), 5 ($F_{MM}=0.15$), 14 ($F_{MM}=0.21$), and 20 min ($F_{MM}=0.31$) and represent a selection of the reaction mechanism analysis data (see Fig. 6). The Ca$^{2+}$ concentration was 1.5 mm. For spectrum denotations, see the legend to Fig. 3.

**Fig. 6.** Triad fractions versus degree of conversion for epimerization of alginate by AlgE4. Reactions were terminated within a time period of 5–120 min. $\triangle$, experimentally determined triad fractions $F_{MM}$, and $F_{GM}$, respectively. Solid lines, long dashed lines, and short dashed lines show calculated triad fractions for the random attack model, the processive model with processivity $=10$, and the preferred attack model using a $K_{MM}/K_{MG}$ (ratio of binding constants) $=25$, respectively. For model descriptions, see under “Discussion.” The statistical variation with regard to interpretation of the nmr spectra was smaller than the symbol areas.
leaves its substrate after each single epimerization event and then preferentially attacks M residues with G residues located as the next nearest neighbor (preferred attack model) (44). The simulation results prove that both the processive and preferred attack models agree better with the experimentally determined triad fractions than the random attack mode (Fig. 6). With the assumption that 10 residues on average are converted per attack, the processive model is in reasonable agreement with the experimental data (Fig. 6). Likewise, a good agreement with experimental data is obtained when assuming that the enzyme binds GMMM sequences over MMMM sequences by a factor of 25. Both the processive and the preferred attack models offer possible explanations for the introduced sequence patterns and are valid also for the potentially reversible reaction. However, evidence to discriminate between the two models is lacking at present. If the processive model turns out to be true, it would be particularly interesting, because it would represent the first known example, to our knowledge, of an enzyme with a mechanism such as this on a polysaccharide substrate. Further experimental work to clarify these issues, in addition to the obvious need for structure determination by x-ray crystallography, could involve the use of substrates of various and alternating sequences.

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