The Azotobacter vinelandii Mannuronan C-5-Epimerase AlgE1 Consists of Two Separate Catalytic Domains*

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The Azotobacter vinelandii enzyme AlgE1 is a member of a family of secreted mannanuronan C-5-epimerases. These enzymes convert β-D-mannuronic acid residues (M) to α-L-guluronic acid residues (G) at the polymer level in the industrially important polysaccharide alginate, leading to altered physical and immunological properties of the polymer. The reaction product of AlgE1 was found to be a mixture of blocks of continuous M residues (M-blocks) and blocks containing alternating M and G residues (MG-blocks). The enzyme is dependent on Ca²⁺ for activity, and only Sr²⁺ of those tested was able to replace Ca²⁺ blocked the activity even at low concentrations. algE1 has been divided into two parts based on the modular type of structure previously reported to be a characteristic of the secreted epimerases, and each part has been expressed in Escherichia coli. These experiments showed that AlgE1 contains two catalytic domains, AlgE1-1, which introduces both M-blocks and MG-blocks, and AlgE1-2, which only introduces MG-blocks. AlgE1-1 has a much lower specific activity than both AlgE1-2 and AlgE1. However, the two halves of AlgE1 seem to cooperate in such a way that they contribute approximately equally to the overall epimerization reaction.

Alginate is a biopolymer synthesized by brown algae and by some bacteria belonging to the genera Azotobacter and Pseudomonas (1–4). It is widely used in industry due to its viscosifying and water binding capacity and the ability to form thermo-stable gels with divalent cations (5). It is also used for immobilization and encapsulation of cells and enzymes (6).

All linear polymers consisting of 1,4-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) are designated alginate. Bacterial alginites may also be O-acetylated at the 2 and/or 3 position of the M residues (7). Since the M residues prefer the 4C1-conformation, while G residues prefer the 1C4-conformation, the relative amount and distribution of G residues have a large effect on the structure and properties of the polymers (8). Alginate does not contain G-blocks and is thus unable to form ionic gels (7). Vegetatively growing cells of Azotobacter vinelandii produce capsular alginate containing mostly M- and MG-blocks. The bacterium can also differentiate into a resting cell called cyst, in which the cell is surrounded by a Ca²⁺-alginate gel rich in G-blocks (9).

Unlike most other heteropolymers alginate is synthesized from only one sugar nucleotide, GDP-M, which is the precursor of mannanuronan. The G residues are then introduced at the polymer level by the enzyme mannanuronan C-5-epimerase. Genes encoding periplasmic epimerases (AlgG) have been cloned from both Pseudomonas aeruginosa and A. vinelandii (10, 11). In addition we have cloned and expressed five genes encoding a family (AlgE1–5) of secreted, calcium-dependent epimerases from A. vinelandii (12). We have shown that at least two of these epimerases generate alginates with different distribution of G-residues. By controlling the expression of the epimerases, the organism can therefore potentially determine the structure of its alginate.

AlgE1–5 are composed of two types of modules, one or two A-modules of 385 amino acids each, and one to seven R-modules containing about 153 amino acids each. The modules within each of the two groups are strongly related at the amino acid level (12). The modular structure of AlgE1 is ARRRAR (Fig. 1). We show here that the N-terminal and C-terminal parts of this enzyme (ARRR and AR, respectively) are both enzymatically active and can be expressed separately in Escherichia coli. The ARRR polypeptide introduces a mixture of MG-blocks and G-blocks, while the AR domain only generates MG-blocks.

 experImental procedures

growth of bacteria—E. coli JM109 (13) was used in this study. For site-specific mutagenesis, the strain BMH71-18 mutS (Promega) was also used. Bacteria were grown at 37 °C in L broth (10 g/liter tryptone, 5 g/liter yeast extract, 5 g/liter NaCl) with shaking or on L-agar supplemented with 100 μg/ml ampicillin when appropriate. For enzyme purifications the cells containing plasmid were grown in a medium containing 30 g/liter tryptone, 15 g/liter yeast extract, and 5 g/liter NaCl.

standard laboratory methods—standard recombinant DNA procedures and protein gel electrophoresis were performed according to Sambrook et al. (14). Transformations were performed according to Chung et al. (15). Protein concentrations were measured using the Bio-Rad Coomassie Brilliant Blue based protein assay, using bovine serum albumin as standard.

constructions of plasmids—AlgE1 was previously expressed from the plasmid pHE16, in which translation initiation might potentially take place both from lacZ' and from the start codon in algE1 mRNA. To

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¶ The abbreviations used are: M residue, β-D-mannuronic acid residues; G residue, α-L-guluronic acid residues; PAGE, polyacrylamide gel electrophoresis; MOPS, 4-morpholinepropanesulfonic acid.
avoids any production of a fusion protein and to facilitate purification of the corresponding protein, we constructed an NcoI site at the start ATG of algE1 without changing the coding sequence of the gene. Site-specific mutagenesis was done by using the pSelect system from Promega Inc. using the oligonucleotide 5’-ACGTGTGAAATCCAGTGCTGCCTTTCCA- ATG3’ (changed bases are underlined). The desired bases are underlined for the mutagenesis. The plasmid could then be subcloned into the ATG expression vector pTrc99A (16), generating plasmid pH11.

The plasmid encoding the first part of the protein, pH37, was constructed by removing the last 1.8 kilobases of the gene using Styl. The HindIII site in the polylinker was then made blunt using Klenow DNA polymerase and religated in order to minimize the C-terminal fusion. The resulting protein (AlgE1-1) has a C-terminal tail of 7 vector-encoded amino acids (STCHRHA).

pHE56 encoding the second part of the protein (AlgE1-2) was constructed by replacing a 3.3-kilobase NcoI-BglII DNA fragment with a 0.8-kilobase AflIII-BglII DNA fragment generated from polymerase chain reaction amplification of the deletion encoding the second A-module of AlgE1. Polymerase chain reaction was performed using the TaKaRa LA polymerase chain reaction kit version 2, with 1 mM MgCl2 in the reaction buffer using the primers 5’-TCTTGGAGCCTCAGTGT- TCAATGCC-3’, binding to the start of the second A-module in AlgE1 and 5’-CTCAAGCTTCTGGCTCCTGGCAGCTT-3’, which binds to the 3’-end of the same A-module. The changed bases in the first primers are underlined, and the effect of this mutation is to introduce an AflIII site and change the start of this A-module from Val to Met.

Measurement of Epimerase Activity by Radioisotope Asssay and by NMR Spectroscopy—Epimerase activities were quantified by measuring the liberation of tritium from 5-3H)galactose in water as described previously (11). Briefly, enzyme extracts, CaCl2, and MOPS (50 mM, pH 6.9) in a total volume of 550 μl was prewarmed at 37 °C. Fifty μl of prewarmed (5-3H)galactose (5 μM/ml in water, specific activity about 100,000 dpm/μg) was added, and the mixtures were incubated further. The reactions were stopped by adding 15 μl of NaCl (5 M) and 800 μM isopropyl alcohol and incubated at ~50 °C for at least 15 min. After centrifugation for 30 min, the radioactivity in 1 ml of the supernatant was measured in a liquid scintillation analyzer. Reaction mixtures containing no enzyme were used as blanks, and all reactions were performed in duplicate. The incubation times and quantities of enzyme were varied to ascertain that the measurements were within the linear part of the assay. CaCl2 was added at a concentration of 3 mM unless otherwise stated.

The epimerizations of alginate for NMR spectroscopy were performed using 7.5 μg of unlabeled alginate in a volume of 6 ml of the same buffer concentrations of CaCl2 are stated for each experiment. Desired degrees of epimerization were achieved by varying the amount of enzyme or the incubation time. To investigate the activity of the epimerases on MG-blocks, alginites which had been epimerized by AlgE2 and AlgE4 were dissolved, and the effect of this mutation is to introduce an AflIII site and change the start of this A-module from Val to Met. AlgE1-2 starts at the second A-module, except that the first amino acid chain reaction amplification of the sequence encoding the second A-module of AlgE1-2 was followed by 7 vector-encoded amino acids. AlgE1-2 starts at the second A-module, except that the first amino acid was changed from Val to Met (Fig. 1). The C-terminal end is identical to that of AlgE1.

The three plasmids were used for expression of AlgE1 and its two truncated derivatives AlgE1-1 and AlgE1-2, and the corresponding crude extracts were used for analysis of epimerase activities. These measurements showed that all three proteins are catalytically active (Table I). It was therefore clear that AlgE1 is a bifunctional enzyme, and that the activities of each part could be analyzed separately.

Purification of AlgE1, AlgE1-1, and AlgE1-2—Initial biochemical characterization of AlgE1 showed that this enzyme displayed a maximum activity at pH 6.9, and at a temperature close to 37 °C (results not shown). These conditions were used throughout the study for all three enzymes. AlgE1 displayed the highest specific activity in the crude extracts (0.14 units/mg of total protein), while the other two had fairly similar specific activities (Table I). Interestingly, this did not correlate well with the amount of protein expressed, since SDS-PAGE analysis (Fig. 2, lanes 1, 3, and 5) demonstrated that the expression level of AlgE1-2 is rather low. These data therefore indicated that the specific activity of purified AlgE1-1 must be low compared with those of whole AlgE1 and AlgE1-2.

The three enzymes were purified by the use of three column chromatography steps. In the first step (ion exchange) the specific activities increased strongly for AlgE1 and AlgE1-2 (Table I). In the next step (hydrophobic interaction) the specific activities increased slightly for AlgE1-2 and slightly for AlgE1, while it was severely reduced in the case of AlgE1-1. This reduction is due to the particularly high sensitivity of this enzyme to ions carried over from the elution buffer (see below). During the final step (gel filtration) the activity of AlgE1-1 was restored, while the specific activity of AlgE1-2 was reduced. The reduction in specific activity may be caused by some degree of enzyme inactivation, but we still decided to keep this step since SDS-PAGE analysis indicated that some minor contaminating proteins were removed (results not shown). The purification ranged from a factor of 36 (AlgE1-1) to a factor of 128 (AlgE1-2), and the purity of the final fractions were found to be satisfactory for further biochemical characterization in all three cases (Fig. 2, lanes 2, 4, and 7).

The specific activities of the purified AlgE1 and AlgE1-2 were similar, while the specific activity of AlgE1-1 was an order of magnitude lower (Table I). We tried to obtain values for the kinetic parameters, but the Km of all three enzymes seemed to be too close to the present detection limit of the assay (6 × 10^-6 M) to obtain reliable results. The values obtained for the reac-


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### Table I

| Purification of AlgE1, AlgE1-1, and AlgE1-2 | Total protein (mg) | Total activity (unit) | Specific activity (unit/mg) | Recovery (%) | Purification fold |
|-------------------------------------------|--------------------|----------------------|----------------------------|--------------|------------------|
| AlgE1                                    |                    |                      |                            |              |                  |
| Crude extract                             | 55                 | 7.5                  | 0.14                       | 100          | 1                |
| Ion exchange                              | 2.6                | 11.2                 | 4.3                        | 149          | 32               |
| Hydrophobic                               | 1.5                | 7.3                  | 4.9                        | 97           | 36               |
| Gel filtration                            | 1.2                | 5.7                  | 4.9                        | 76           | 36               |
| AlgE1-1                                   |                    |                      |                            |              |                  |
| Crude extract                             | 46                 | 0.96                 | 0.021                      | 100          | 1                |
| Ion exchange                              | 0.9                | 0.42                 | 0.11                       | 44           | 5.2              |
| Hydrophobic                               | 1.7                | 0.024                | 0.014                      | 2.5          | 0.67             |
| Gel filtration                            | 0.42               | 0.155                | 0.37                       | 16           | 18               |
| AlgE1-2                                   |                    |                      |                            |              |                  |
| Crude extract                             | 76.5               | 2.6                  | 0.034                      | 100          | 1                |
| Ion exchange                              | 0.39               | 1.02                 | 2.6                        | 39           | 76               |
| Hydrophobic                               | 0.11               | 0.91                 | 8.2                        | 35           | 237              |
| Gel filtration                            | 0.11               | 0.48                 | 4.4                        | 18           | 128              |

*One unit is defined as the amount of enzyme needed to epimerize 1 µmol of substrate (sugar residues) in 1 min.

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...tion rates in these experiments indicated that the maximal rates were reached already at a substrate concentration of about 1 \( \times 10^{-4} \) M and that the reaction was not significantly inhibited at the substrate concentrations used in the standard assay (results not shown). This implies that the specific activities shown in Table I are proportional to the \( k_{\text{cat}} \) of each enzyme.

The molecular masses of the epimerases seemed to be somewhat higher based on the SDS-PAGE compared with the values deduced from the DNA sequences (162, 97, and 77 kDa compared to 147, 89, and 59 kDa for AlgE1, AlgE1-1, and AlgE1-2, respectively). This is similar to what has been previously observed for an AlgE-epimerase isolated from *A. vinelandii* (18), and thus probably is not related to expression of the protein in a foreign host. It has been reported that calcium-binding proteins may migrate aberrantly in SDS-PAGE gels, but that the relative migration rates can be changed by adding calcium to the sample buffer (19). In our case, however, the apparent molecular masses of the three proteins were not affected by addition of 1 mM CaCl\(_2\) or 1.7 mM Na\(_2\)EDTA to the sample buffer, gel, and separation buffer (results not shown). The purified AlgE1 (lane 2) still shows two bands on the gel. We have observed that several of the epimerases are seen as multiple bands on denaturing gels, although in the present case the possibility of a contaminating protein has not been ruled out.

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The Effects of the Salts in the Purification Buffers on Epimerase Activity—During purification of the enzymes we observed that the activities might be strongly affected by the presence of the NaCl and (NH\(_4\))\(_2\)SO\(_4\) used in the protocol. These effects were therefore analyzed more systematically. The data in Fig. 3A show that a concentration between 100 and 200 mM NaCl was optimal for AlgE1, AlgE1-1, on the other hand, is much more sensitive to high ionic strengths than whole AlgE1, but also compared with AlgE1-2. Because of the shapes of the different activity curves there is actually no concentration that is near optimal for all three enzymes.

The effects of (NH\(_4\))\(_2\)SO\(_4\) seemed to be quite dramatic during the purification of AlgE1-1. As can be seen from Fig. 3B, the activity of all three enzymes is quite sensitive to this salt. Note that a concentration of about 30 mM exerts a very significant stimulatory effect on AlgE1 and AlgE1-2, while the same concentration strongly represses the activity of AlgE1-1. When the activity data from the purification of AlgE1-1 were adjusted for the positive effects of NaCl and the negative effects of (NH\(_4\))\(_2\)SO\(_4\), the enzyme preparations from the hydrophobic column and the gel filtration column both have a total activity of 0.11 units. This therefore also explains the apparently very low specific activity obtained for AlgE1-1 after the hydrophobic interaction chromatography step.

The Effect of Calcium on the Activity of the Three Epimerases—It has long been known that the secreted *A. vinelandii* epimerases need Ca\(^{2+}\) for activity, and that different enzymes probably have different requirements for this ion. We therefore decided to study the influence of calcium on AlgE1 and its derivatives. The optimal concentration of Ca\(^{2+}\) for AlgE1 activity was found to be 0.8 mM (Fig. 3C). At 0.05 mM CaCl\(_2\) no activity was detected, while 0.35 mM was required to obtain 50% of the maximal activity. At concentrations above optimum the activities were somewhat, but not severely, reduced. The data in Fig. 3C also show that the Ca\(^{2+}\) requirement of AlgE1-1 is much higher (optimum around 3 mM) than that of whole AlgE1, while AlgE1-2 needs slightly more Ca\(^{2+}\) than AlgE1 for optimal activity. It is also interesting to note that the slope of the AlgE1-1 curve is steep up to about 25% of maximal activity (0.2 mM Ca\(^{2+}\)), and then is much more moderate at higher Ca\(^{2+}\) concentrations. AlgE1-1 therefore has a higher relative activity than AlgE1-2 at low concentrations of calcium. The purification data shown in Table I were all determined in the presence of 3 mM Ca\(^{2+}\), and this may have led to an underestimation of the activities during purification for AlgE1 and to a lesser extent also for AlgE1-2.

The Effect of Other Divalent Cations on AlgE1—In the presence of the Ca\(^{2+}\) chelator Na\(_2\)EDTA, AlgE1 activity was, as expected, severely reduced or blocked. Still, as long as the molar Na\(_2\)EDTA concentration was kept below that of Ca\(^{2+}\), the enzyme activity was not eliminated. It is therefore clear that the inhibitory effect of Na\(_2\)EDTA is directly caused by its ability to bind divalent cations. Interestingly, Sr\(^{2+}\) was able to substitute for Ca\(^{2+}\), although the activity was not fully restored (results not shown). At Ca\(^{2+}\) concentrations lower than optimum, the addition of Sr\(^{2+}\) or Mg\(^{2+}\) had a stimulatory effect (Fig. 4), but Mg\(^{2+}\) is not able to substitute for Ca\(^{2+}\) when no calcium is added (results not shown). These results are in accordance with those found by Haug and Larsen (20) for a mixture of epimerases isolated from *A. vinelandii*. Both Mn\(^{2+}\) and Zn\(^{2+}\) exert a negative effect on enzyme activity, the effect of Zn\(^{2+}\) being strongly negative even at rather low concentrations (0.1 mM).
by the Three Epimerases—The monomer sequence distribution of the epimerase reaction products were determined by NMR spectroscopy. The results showed that AlgE1 is able to introduce both G-blocks and MG-blocks into its substrate (Fig. 5A). The properties of AlgE1 therefore seem to include those previously reported for both AlgE2 and AlgE4 (12, 18). The effect of the Ca²⁺ concentration on the epimerization pattern was also analyzed, and the results showed that the relative amount of G-blocks at a given FG increases with decreasing amount of calcium down to 0.4 mM (Fig. 6). Furthermore, as the degree of epimerization increases at a given Ca²⁺-concentration (0.4 and 3.0 mM tested), the relative frequency of G-blocks increases (Fig. 6). This increase is presumably due to elongation of pre-existing G-blocks as the reaction proceeds. The addition of 100 mM NaCl to the epimerization reaction did not significantly alter the relative content of G-blocks and MG-blocks (Table II, samples 2 and 3).

A similar analysis carried out with AlgE1-1 and AlgE1-2 showed that AlgE1-1 introduces G into both G-blocks and MG-
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blocks, with a preference for G-blocks (Fig. 5B), while AlgE1-2 only generates MG-blocks (Fig. 5C). Thus it can be concluded that each catalytic center in AlgE1 catalyzes the formation of very different alginate structures, and that AlgE1-2 displays an activity that is very similar to that of AlgE4. The frequency of G-blocks in the reaction product of whole AlgE1 is quite high despite the very low specific activity of the part apparently responsible for this activity (AlgE1-1). The simplest explanation for this inconsistency is probably that the catalytic efficiencies of the two separate halves of the enzyme are different from their activities in the fused polypeptide (AlgE1). We also mixed equimolar quantities of AlgE1-1 and AlgE1-2 and measured the monomer sequence distribution in the corresponding reaction product (Table II, samples 12 and 13). Interestingly, the relative frequency of G-blocks is higher than one would expect from the specific activity of each enzyme. It is not clear whether this indicates that the activities of the two enzymes are influenced by the presence of each other, or that the product of one enzyme is a more favored substrate for the other than is the original substrate.

Another interesting question is whether the enzyme is able to epimerize M residues flanked by G residues. To analyze this problem we incubated an AlgE4 reaction product containing only M-blocks (20%) and MG-blocks (80%) with the three enzymes. AlgE1-2 was not able to epimerize this substrate further, while AlgE1 and AlgE1-1 were able to introduce some G-residues as G-blocks (Table II, samples 4, 8, and 11). Since the fraction of GM did not decrease, the enzymes do not seem to be able to epimerize the M in the triad GMG.

**DISCUSSION**

The results presented in this report clearly demonstrate that AlgE1 has two active centers, one generating MG-blocks only, while the second preferentially generates G-blocks. AlgE1 therefore can be considered functionally as a fusion of an AlgE2-like and an AlgE4-like protein, except that AlgE1-1 introduces a higher proportion of MG-blocks than AlgE2 (12). A. vinelandii might need such an epimerase to synthesize alginate containing a mixture of G-blocks and MG-blocks. This may be more difficult to achieve if two separate enzymes, each introducing different types of block structures, were used. One might also envision that the G-blocks in the reaction product of AlgE1 function as junction zones in a gel network, while the other block types serve as elastic elements between the cross-links. At low concentrations of Ca\(^{2+}\) the average length of the G-blocks needs to be higher to maintain a constant gel strength (21). Interestingly, AlgE1 introduces relatively more G-blocks when the concentration of Ca\(^{2+}\) is 0.4 mM than when it is 3 mM. This could be caused by a shift in the relative activity of the two functional units when the concentration of Ca\(^{2+}\) is changed. The data in Fig. 3C are consistent with this hypothesis, since they indicate that the N-terminal half of AlgE1 displays relatively more activity at low concentrations of Ca\(^{2+}\) than the C-terminal part.

When G-block forming epimerases are used to epimerize alginates, it is always a problem that aggregates of alginate molecules cross-linked with Ca\(^{2+}\) are formed. Since the binding of Ca\(^{2+}\) competes with the binding of Na\(^{+}\) and Mg\(^{2+}\), neither of which is able to cross-link G-blocks, addition of sufficient amounts of these cations to the reaction mixture should theoretically reduce this problem. The results shown in Fig. 3A show that AlgE1 actually is activated by up to 200 mM NaCl, while AlgE1-1 tolerates only up to 100 mM NaCl. The addition of NaCl does not seem to influence the epimerization pattern of AlgE1. Since in addition the concentration of Ca\(^{2+}\) needed for full activity can be lowered when Mg\(^{2+}\) is added (Fig. 4), it

**TABLE II**

| Sample | Enzyme          | \(F_G\) | \(F_{GG}\) | \(F_{GM}\) | \(F_{GG}/F_{GM}\) | Comments          |
|--------|-----------------|--------|-----------|-----------|-------------------|-------------------|
| 1      | AlgE1           | 0.294  | 0.103     | 0.191     | 0.54              | 0.4 mM Ca\(^{2+}\), 100 mM NaCl |
| 2      | AlgE1-1         | 0.264  | 0.137     | 0.127     | 1.08              | 100 mM NaCl       |
| 3      | AlgE1-2         | 0.483  | 0.253     | 0.229     | 1.10              | MG-alginate       |
| 4      | AlgE1–1         | 0.421  | 0.030     | 0.391     |                   |                   |
| 5      | AlgE1–2         | 0.189  | 0.096     | 0.093     | 1.03              |                   |
| 6      | AlgE1–1         | 0.292  | 0.169     | 0.123     | 1.37              |                   |
| 7      | AlgE1–2         | 0.489  | 0.358     | 0.131     | 2.7               |                   |
| 8      | AlgE1–1         | 0.456  | 0.059     | 0.397     |                   |                   |
| 9      | AlgE1–2         | 0.293  | 0        | 0.293     | 0                 |                   |
| 10     | AlgE1–1         | 0.348  | 0        | 0.348     | 0                 |                   |
| 11     | AlgE1–2         | 0.405  | 0        | 0.405     |                   |                   |
| 12     | AlgE1–1 + AlgE1–2 | 0.294  | 0.073     | 0.221     | 0.33              |                   |
| 13     | AlgE1–1 + AlgE1–2 | 0.351  | 0.121     | 0.230     | 0.53              |                   |

\(^a\) Equimolar amounts of AlgE1–1 and AlgE1–2, 16 times less AlgE1–1 than in sample 5 and half the amount of AlgE1–2 as compared to sample 9.  
\(^b\) Twice as much of each enzyme as in sample 12.
should be possible to compose a reaction buffer which ensures both high activity and that a higher $F_{G}$ can be reached before the problem of aggregation occurs. The results also seem to suggest that $Ca^{2+}$ has two different functions in the reaction, and that one of those are more ion-specific than the other. The first part of the epimerization reaction is proposed to be neutralization of the negatively charged mannuronic acid residue (22). Any cation bound to the alginate would be expected to make this step easier. The epimerases contain sequences homologous to known calcium-binding motifs (11), thus the enzyme probably needs to bind either $Ca^{2+}$ or $Sr^{2+}$ to be active, with $Ca^{2+}$ being the preferred ion.

The data seem to suggest that AlgE1-1 has a much lower specific activity alone than when it is part of the whole enzyme, while this is not the case for AlgE1-2. This may be caused by incorrect folding since the C terminus of AlgE1-1 is not similar to the termini of the epimerases found in A. vinelandii. The NMR spectra obtained by using a mixture of AlgE1-1 and AlgE1-2 seem, on the other hand, to indicate that mixing of the two halves stimulates the activity of AlgE1-1. It is therefore possible that the two halves can associate with each other and that this interaction is necessary for full activity of the first part. It may also be that AlgE1-1 needs pre-existing G-residues to be able to epimerize efficiently. It should then be expected that the relative amount of G-blocks introduced by AlgE1 increases with increasing $F_{G}$, and this is consistent with the data in Fig. 6.

The experiments using MG-alginate as substrate suggest that AlgE1 and AlgE1-1 are able to epimerize mannuronic acid residues which are not accessible to AlgE1-2. This could imply that they are able to use smaller M-blocks as substrate, perhaps because they are able to epimerize an M next to a G. However, since the epimerization capacity is quite low on this substrate, the smallest accessible M-block must consist of more than one residue, probably also more than two. AlgE1 is therefore not able to convert MG-blocks to G-blocks.

As part of an attempt to better understand the biological function of the epimerases in A. vinelandii, it will be necessary to analyze the structures and properties of the reaction products of all the enzymes. It seems very probable that the need for varying the physical properties of the alginites is related both to the vegetative and cyst state of the cells. By also carrying out expression studies of the epimerase genes under different environmental conditions, combined with gene knock-out studies, it should eventually be possible to understand the major aspects of this rather unique biological system. Experiments along these lines have therefore been initiated in our laboratory.

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