The Crystal Structure of Pectate Lyase Pel9A from Erwinia chrysanthemi

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The “family 9 polysaccharide lyase” pectate lyase L (Pel9A) from Erwinia chrysanthemi comprises a 10-coil parallel β-helix domain with distinct structural features including an asparagine ladder and aromatic stack at novel positions within the superhelical structure. Pel9A has a single high affinity calcium-binding site strikingly similar to the “primary” calcium-binding site described previously for the family Pel1A pectate lyases, and there is strong evidence for a common second calcium ion that binds between enzyme and substrate in the “Michaelis” complex. Although the primary calcium ion binds substrate in subsite −1, it is the second calcium ion, whose binding site is formed by the coming together of enzyme and substrate, that facilitates abstraction of the C5 proton from the saccharide in subsite +1. The role of the second calcium is to withdraw electrons from the C6 carboxylate of the substrate, thereby acetylenizing the C5 proton facilitating its abstraction and resulting in an E1cb-like anti-β-elimination mechanism. The active site geometries and mechanism of Pel1A and Pel9A are closely similar, but the catalytic base is a lysine in the Pel9A enzymes as opposed to an arginine in the Pel1A enzymes.

Polysaccharide lyases (EC 4.2.2.-) are carbon-oxygen lyases that exploit β-elimination chemistry to cleave C5 uronic acid containing pyranoside substrates such as polygalacturonates, alginates, hyaluronan, and chondroitin. In contrast to the 90 sequence-derived families of glycoside hydrolases, polysaccharide lyases can be grouped into 13 sequence-based families (1). Three-dimensional structures have been reported for enzymes from polysaccharide lyase families (PL) families 1, 3, 5, 6, 8, and 10, and of these, PL-1 (9–5), PL-3 (6), and PL-6 (7) consist of “right-handed parallel β-helix” domains (called “parallel β-helix” domain throughout the rest of this article). The parallel β-helix domain is superhelical and comprises three short β-strands, typically three to four residue, per coil of the superhelix; these coils stack coil-on-coil to form three parallel β-sheets that extend the whole length of the domain, and these β-sheets are referred to as PB1, PB2, and PB3. Schematic diagrams of the three parallel β-helix domains, including that of Pel9A reported in this report, are shown in Fig. 1. The turns between the short β-strands are known as T1, T2, and T3, and these turns connect PB1 to PB2, PB2 to PB3, and PB3 to PB1 of the next coil of the superhelix, respectively (see Fig. 2a).

Glycoside hydrolases (EC 3.2.1.-) hydrolyze the carbon-oxygen bond between two pyranoside residues or between a pyranoside and another moiety. The three-dimensional structures of the solved glycoside hydrolases show more structural diversity, although the β/α barrel occurs frequently, often with catalytic carboxylates at the ends of β-strands 4 and 7 (8, 9). So far, only one glycoside hydrolase family has been shown to have parallel β-helix architecture, family GH-28 (10), a family that includes the enzyme polygalacturonase (11), although three other families have been predicted as parallel β-helices (12). Carbohydrate esterases can be grouped into 13 sequence-based families, and the parallel β-helix fold occurs again in carbohydrate esterase family CE-8, a family of pectin methyl esterases (13). The parallel β-helix fold of each of these families has its distinct structural features (14).

Pectate lyases cleave polymeric α-1,4-linked galacturonic acids (GaLa) of the pectate component of the plant cell wall by a β-elimination mechanism that generates a 4,5-unsaturated oligogalacturonate product. All pectate lyases, with the exception of PelW (15), require calcium for in vitro activity and presumably utilize the abundant calcium in the plant cell wall for activity in vivo. The role of calcium has been controversial, but it is now clear that it binds to both enzymes and substrates and can mediate enzyme-substrate interactions by binding between enzyme and substrate (16). Some pectate lyases have been shown to have a preference for partially methylated polymers of polygalacturonic acid, and the non-calcium-dependent pectin lyases that cleave fully methylated substrates also belong to family PL-1 (17).

Erwinia chrysanthemi is a pathogenic enterobacterium that causes soft-rot diseases in a variety of crops. The bacterium enters the plant through wounds and secretes polygalacturonase-active enzymes that break down the complex polysaccharide pectin. Pectin is a key component of the plant cell wall, and these enzymes enable the further invasion of the bacterium. E. chrysanthemi is known to produce six PL-1 (PelA, -B, -C, -D, -E, and -F), a PL-2 (PelW), a PL-3 (PelI), and two PL-9 (PelIL and PelIX) pectate lyases. PelIL is an endo-acting, calcium-dependent pectate lyase with relatively low activity against ploygalacturonate (~100-fold less than the major PL-1 pectate lyase).

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The atomic coordinates and structure factors (code 1RU4) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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Carbohydrate-Active Enzymes server, afmb.cnrsmrs.fr/−pedro/CAZY/db.html.

The abbreviations used are: PL, polysaccharide lyase; GaLa, galacturonic acids; Pel, pectate lyase; BsPel, Bacillus subtilis Pel.
lyase PelB) and with a pH optimum around 8.0 (18, 19).

Here we report the 1.6-Å resolution crystal structure of the endo-acting pectate lyase L (EC 4.2.2.2) from E. chrysanthemi 3937 (called Pel9A throughout this article as it is a paradigm for the polysaccharide lyase family 9 enzymes). The fold of Pel9A was correctly predicted as part of the Critical Assessment of Techniques for Protein Structure Prediction competition (20), but the modelers had severe difficulties producing a molecular model for Pel9A because of the repetitive nature of the fold, the short β-strands, and the many possibilities for excursions between the β-strands. When the “primary” calcium-binding sites of the previously described Pel1A and the Pel9A structure described here are superimposed, the active site geometry is seen to be closely similar, but lysine in Pel9A replaces the catalytic arginine of the Pel1A enzymes. The K273A mutant of Pel9A is inactive but correctly folded, supporting the assignment of lysine 273 as the catalytic base.

### MATERIALS AND METHODS

**Production of Native Pectate Lyase L**—The pectate lyase L gene from E. chrysanthemi (PelL) was overproduced in Escherichia coli BL21 cells and purified from the periplasm by ion-exchange and gel filtration chromatography (21). The purified protein gave a single band on Coomassie Blue-stained SDS-PAGE gels. The protein was concentrated to 20–30 mg/ml for crystallization, and the largest crystals grew from 16 to 17% 1,6-hexanediol, 12% H2O855/H9252, and with a single molecule in the asymmetric unit.

**Preparation of Heavy Atom Derivatives and Data Collection**—Data were collected from crystals cooled to 100 K in the nitrogen gas stream of an Oxford Cryosystems cryoloop using synchrotron radiation and image plate or CCD detector (Table I). No additional cryoprotectant was needed for successful flash freezing for native or heavy atom soaked crystals. Heavy atom derivatives were prepared by soaking Pel9A crystals in the heavy atom solutions described in a previous work (22). PelL (Pel9A) was for generating heavy atom derivatives, lead acetate, and sodium hexachloroiridate. The concentrations and soak times used are given in Table I as is a summary of the crystallographic statistics. Data were reduced using DENZO and SCALEPACK (22), and subsequent calculations used CNS (23) and the CCP4 program suite (24).

**Structure Solution**—Inspection of the platinum orange difference Patterson revealed a single platinum-binding site. The correct hand of the solution was that which gave the greatest peak heights on cross-phased difference Fourier maps. The heavy atom positions from the difference Patterson and the difference Fourier maps were refined using MLPHARE (24), and protein phases were calculated to a 2.3-Å resolution. DM (25) was then used to improve the protein phases, and the difference Fourier maps were recalculated, reviewed, and re-refined. DM was then run at 1.6 Å to give protein phases that were used in WARP (27). WARP successfully built 85% of the protein model. Refinement was completed using CNS and REFMAC (28) combined with model building using O (29). PROCHECK was used to judge the stereochemical quality of the final structure (30). Sequence alignments were used T-Coffee (31). Crystallographic statistics are given in Table I. The presence of a calcium ion at the active site was confirmed by inspection of an anomalous difference map.

**Construction and Analysis of Mutant Proteins**—The single mutations K273A and K273R were introduced in PelL (Pel9A) by site-directed mutagenesis with the QuikChange kit (Stratagene). The primers K273A (5′-ggcaagggcttggcctagggaac3′) and K273A-re, the reverse complementary to K273A, were used to create K273A, and the primers K273R (5′-ggcaagggcttggcctagggaac-3′) and K273R-re, the reverse complementary to K273R, were used to generate K273R
RESULTS AND DISCUSSION

The Architecture of Pel9A—The three-dimensional structure of Pel9A from *E. chrysanthemi* was solved at a resolution of 1.6 Å (see "Materials and Methods" and Tables I and II). The mature polypeptide chain (residues 26–425) of Pel9A folds to form a right-handed parallel β-helix (residues 95–356) with distinct structural features and with 10 turns of superhelical architecture (Fig. 1). The parallel β-helix domains of Pel1A and Pel3A are shorter than Pel9A with only eight complete superhelical turns but, like polygalacturonase, Pel9A has 10 turns (Fig. 1). When compared with Pel1A, Pel9A has considerably shorter and better-ordered loops. All three enzymes shown in Fig. 1 have an N-terminal α-helix that caps the hydrophobic core of the parallel β-helix.

The β-helix of Pel9A is both preceded and followed by less regular regions, and there is a single disulphide bridge between cysteines 28 and 114 that links the third residue of the N-terminal extension to the beginning of the second β-strand of PB2. The N-terminal extension forms the first strand of PB2 (residues 40–44) and an extravagant but well ordered loop (residues 47–62) before forming the α-helix (residues 62–70) that contributes Phe-63 and Met-67 to the capping of the hydrophobic core at the N-terminal end of the β-helix. There then follow 10 turns of superhelical architecture, starting with the second strand of PB2 (residues 74–77). At the end of the β-helix domain, the polypeptide breaks away abruptly from the final β-strand of PB3 to form the C-terminal extension (residues 357–425) that comprises an extended irregular hairpin that packs against PB3 and further irregular structure, part of which is stabilized by a sodium ion in the crystal structure. Trp-357 of the C-terminal extension terminates the aromatic stack on PB1 (see below) and plugs the hydrophobic core at the C-terminal end of the β-helix.

In comparison with the other right-handed β-helix structures solved, Pel9A has a very regular arrangement of side chains and main chain in the T3 turns (between PB3 of coil n and PB1 of coil n + 1). This regularity is conferred by the remarkably well ordered like-on-like stacks in this region, which includes an eight-residue glycine stack, a six-residue asparagine ladder at T3 (asparagines 174, 198, 231, 255, 293, and 317), and a four-residue alanine stack (alanines 195, 228, 252, and 290) (Fig. 2, a and b). In Pel9A, there are also additional short asparagine ladders within the T2 turn (Asn-312, Asn-338, and Asn-355) and within the T1 turn (Asn-164, Asn-188, and Asn-221). The regularity in the T3 region extends to the main chain conformation that forms hydrogen bonds up and down the length of the parallel β-helix. It is the T2 turn extra sheet is shown in blue, and as it follows PB1, it is referred to as PB1a. Also shown are the active site residues Asp-202 (col 3), Asp-224 (col 6), and Arg-280 (col 8). Figs. 1 and 2 were prepared using MOLSCRIPT (40).
and PB3 that possess the asparagine ladder and the aromatic stack characteristic of the Pel1A enzymes (Fig. 2, c and d). The regular aromatic stack of Pel1A (Fig. 2d) is replaced by the alanine stack described above in Pel9A, and for many turns, the less regular aromatic stack on PB1 fills the parallel \( \beta \)-helix (phenylalanines 211, 236, 272, and 298 and tyrosine 321; Fig. 2b). Although an aromatic residue is shown on PB2 of Pel9A (Fig. 2a), this is not involved in extensive stacks. There is also an external aromatic stack on the surface of PB2 in Pel9A involving His-172, Tyr-196, Trp-229, Phe-253, Phe-291, and Tyr-315. The distinctive features of Pel9A, the aromatic stack on PB1, and the asparagine ladder within the T3 turn are interesting and show how different sequences can pack the core of the \( \beta \)-helix domain. The success of the parallel \( \beta \)-helix in polygalacturonate-active enzymes is presumably because the binding surface on the external surface of PB1 and adjacent loops is well suited to binding polygalacturonates.

**Calcium Binding to Pel9A and Pel1A Enzymes**—A single calcium ion is seen bound to Pel9A (Fig. 3a) despite no addition of calcium during purification and crystallization, which implies that the site has at least \( \mu \)M affinity and that the enzyme has scavenged the calcium present in trace quantities from the solutions. The calcium-binding site is formed by four aspartates on the external surface of PB1. This calcium-binding site is strikingly similar both in its position on the surface of PB1 and in the nature of the calcium ligands to the first calcium site identified both in the Pel1A structures (4, 32) and in the pH 9.5 Pel3A structure (6). Superimposition of the calcium ion of Pel9A with the equivalent calcium in the PelC substrate complex reveals that Asp-131, Glu-166, and Asp-170 of Pel9A correspond to Asp-209, Asp-233, and Asp-237, respectively, of Pel9A. If the common calcium-binding residues are superimposed, then the \( \beta \)-helices are brought into close structural alignment, revealing that, when compared with the Pel1A enzymes, the two additional coils of Pel9A are N-terminal to the calcium-binding site such that in Pel9A, the calcium-bind-
aspartates are on coils 5 and 6, whereas in PelC, they are on coils 3 and 4 (Fig. 3). PelC and Pel9A superimpose with a root mean square deviation of 1.8 Å for 171 equivalent carbon atoms.

Data collected from native Pel9A crystals soaked in substrates and calcium did not reveal bound ligand or additional calcium-binding sites (data not shown), but the Pb sites used to calculate the protein phases do indicate the presence of a second calcium-binding site close to the active center of Pel9A. The primary calcium-binding site is strikingly similar to that of Pel1A (BsPel). The second calcium site is anticipated to bind the complex at a similar position (as suggested by the Pb2 site), and the third and fourth sites will be different if they exist in Pel9A. Phenylalanine 239 in Pel9A forms a platform for the galacturonate in subsite −1. This figure was prepared using BOBSCRIPT (2).

FIG. 3. The calcium-binding sites of Pel9A and Pel1A. a, anomalous difference Fourier map (chicken wire mesh) revealing the presence of a metal bound to Pel9A by aspartates 209, 233, and 237 (Asp-234 is also a calcium-ligand, not shown). The putative catalytic base, lysine 273, is also shown. b, calcium-binding site of Pel1A (BsPel) showing calcium bound by aspartates 184, 223, and 227. The catalytic base, arginine 279, is also shown. c, substrate binding to Pel1A (PelC coordinates kindly provided by Prof. Fran Jurnak). Galacturonates occupying subsites −1 (left) and +1 (right) are shown together with four calcium-binding sites (ligands for sites three and four are not shown). d, substrate modeled into the active center of Pel9A. The primary calcium-binding site is strikingly similar to that of Pel1A (BsPel). The second calcium site is anticipated to bind the complex at a similar position (as suggested by the Pb2 site), and the third and fourth sites will be different if they exist in Pel9A. Phenylalanine 239 in Pel9A forms a platform for the galacturonate in subsite −1. This figure was prepared using BOBSCRIPT (2).
second calcium in the Pel9A-substrate complex (Fig. 3d). The
aspartates contributing to the third and fourth calcium-binding
sites in PelC are not present in Pel9A. However, Asp-299 in
Pel9A may form a calcium-binding site together with substrate
carboxylates, and this calcium would be in a similar position to
the fourth calcium in PelC.

There are no arginines in the substrate-binding cleft of
Pel9A, and so substrate carboxylate interactions with arginin
involved both carboxylate oxygens and hydrogens of NH1
and NH2 nitrogens of the arginine, as seen for the galacturonic
residue at subsite +2 and Arg-245 in PelC, cannot occur in
Pel9A. However, when compared with PelC, Pel9A has two
additional carboxylates, Asp-299 and Glu-180, in the substrate
binding cleft, and these could form calcium-mediated interac
tions with substrate carboxylate oxygens. Asp-299 (coil 8) is
close to the putative base, and Glu-180 (coil 4) is on the leaving
group side of the cleft.

Pel9A has low activity on polygalacturonate (19) but is re
ported to be effective in the maceration of plant tissue (18).
Polygalacturonate is a model substrate, and Pel9A may have
more activity against its true plant cell wall substrate. Alterna
atively, Pel9A may have low activity as it is constitutively
expressed, suggesting its role as a reconnaissance molecule.
Alternatively, Pel9A may have low activity as it is constitutively
expressed, suggesting its role as a reconnaissance molecule.

Catalytic Mechanism—Pel1A and Pel9A enzymes cleave
\( \alpha \)-1,4 bonds of polygalacturonate by anti-\( \beta \)-elimination charac
terized by base-catalyzed abstraction of the proton from C5
and elimination of the substituent at O4 to give an unsaturated
product. \( \beta \)-elimination chemistry is reviewed in Anderson (33).
In the family Pel1A enzymes, it has been argued that the arginine is the
Brønsted base that abstracts the proton from C5 (16). Arginine is a surprising base for an enzyme active at pH
8.0, but calcium ions close to the arginine in the Michaelis
complex could depress its \( pK_a \) (34); as discussed below, the
putative base is lysine in Pel9A as opposed to arginine. There is
no obvious enzyme or substrate Brønsted acid to protonate the
glycosidic oxygen in these enzymes, and protonation is
presumed to occur via solvent water. There are, however,
substrate hydroxyls that could stabilize the departing anion.
Hydrogens from the O3 hydroxyl of the galacturonate in subsite
+1 and from the O2 hydroxyl of the galacturonate in subsite
-1 could stabilize the oxyanion of the leaving group (Figs. 3
and 4). The argument for arginine as base is strongly supported
by the recent Pel10A structure that has convergent active site
genetics with the Pel1A enzymes, including an active site
arginine, but a non-related architecture (35). Perhaps the most
remarkable difference between the active sites of Pel9A and
these other polysaccharide lyases is that in Pel9A, there is no
arginine equivalent to the putative base in Pel9A (Arg-218 in
PelC). If arginine is substituted for lysine in the coordinates of
the inactive PelC complex, then the end of Lys-273 in Pel9A is
close to the end of the Arg-218 in PelC. Because lysine is
shorter than arginine for the ends of the side chains to arrive in
the same location relative to bound substrate, the lysine is
situated on the coil adjacent to the calcium-binding site,
whereas the arginine is on the next but one coil (Fig. 3). When
Lys-273 was mutated to alanine, no activity could be detected,
a result consistent with lysine acting as the base. When Lys-
273 was mutated to arginine, the activity was less than 5% that
of the native enzyme, suggesting that arginine is capable of
catalyzing the reaction but is not in an optimal position for
catalysis. Lys-296 was also a candidate for the base in Pel9A,
(33). The E2 pathway describes concerted but not necessarily synchronous proton abstraction and leaving group elimination such as would occur if the lifetime of the carbanion is less than the vibrational frequency of the Cβ-X bond (39).

The presence of a putative catalytic base (lysine 273) in Pel9A, but no clear catalytic acid, suggests that the favored reaction pathway will be E1cb or toward E1cb rather than E1 on the More O’Ferrall potential energy diagram. The calcium ion interaction with the substrate carboxylate at C5 will increase the acidity of the C5 proton, and this, too, favors cleavage of the Cα-H bond, again favoring an E1cb mechanism (Fig. 4b). Substrate hydroxyls may facilitate leaving group departure.

There are three possible reasons why Pel9A is less active on polygalacturonates and oligogalacturonates than the Pel1A enzymes. Firstly, the lysine of Pel9A is a less potent base than arginine. Secondly, although there is strong evidence that the C5 proton may be absent or at least not in the same position as in PelC. Thirdly, polygalacturonase may be a poor substrate for Pel9A.

**Comparison of Pel9A and Pel1A Lyases with Polygalacturonase**—When polygalacturonase has 10 turns of β-helix, as does Pel9A, and when polygalacturonase is superimposed coil-for-coil on Pel9A, then two of the putative active site residues of polygalacturonase Asp-202 and Asp-224 are superimposed on two of the calcium-binding residues Asp-209 and Asp-234 of Pel9A. When compared with Pel1A enzymes, not only are the aspartates in the same position in polygalacturonase, but also the arginine, the putative base in the Pel1A enzymes (32). Not only is the chemistry different in polygalacturonase, but also, a different bond is broken. In the hydrolase, it is the C1 O bond. It is fascinating to see a common topology and the related active site geometries in these enzymes giving rise to different catalytic residues (Pel9A when compared with Pel1A) and different active site chemistries (lyases as compared with hydroxylase).

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