Characterization and Implications of Ca\(^{2+}\) Binding to Pectate Lyase C*

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Ca\(^{2+}\) is essential for in vitro activity of *Erwinia chrysanthemi* pectate lyase C (PelC). Crystallographic analyses of 11 PelC-Ca\(^{2+}\) complexes, formed at pH 4.5, 9.5, and 11.2 under varying Ca\(^{2+}\) concentrations, have been solved and refined at a resolution of 2.2 Å. The Ca\(^{2+}\) site represents a new motif for Ca\(^{2+}\), consisting primarily of \(\beta\)-turns and \(\beta\)-strands. The principal differences between PelIC and the PelC-Ca\(^{2+}\) structures at all pH values are the side-chain conformations of Asp-129 and Glu-115. Additional structural changes lower the pKa of Arg-218, the amino acid responsible for proton abstraction during catalysis. The Ca\(^{2+}\) affinity for PelC is weak, as the K\(d\) was estimated to be 0.132 (±0.004) mM at pH 9.5, 1.09 (±0.29) mM at pH 11.2, and 5.84 (±0.41) mM at pH 4.5 from x-ray diffraction studies and 0.133 (±0.045) mM at pH 9.5 from intrinsic cryptophan fluorescence measurements. The pH dependence of Ca\(^{2+}\) affinity of PelC activity at pH 4.5 has been reexamined. At saturating Ca\(^{2+}\) concentrations, PelC activity increases 10-fold at pH 4.5 but is less than 1% of maximal activity at pH 9.5. Taken together, the studies suggest that the primary Ca\(^{2+}\) ion in PelC has multiple functions.

*Erwinia* sp. are pathogenic bacteria that are causally involved in soft rot diseases in a wide variety of plant crops (1, 2). The bacteria enter the plant through wounds produced by either insects or harvesting and subsequently multiply in the intercellular spaces, secreting large amounts of pectolytic enzymes. These enzymes degrade the middle lamella portion of the plant cell wall, causing tissue damage and cell rupture. *Erwinia* bacterial strains, which are deficient in one or more pectate lyase genes, have decreased virulence (3–5). Mutations affecting the extracellular secretion of the pectic enzymes also reduce virulence (6). The introduction of pectate lyase genes into *Escherichia coli* enables *E. coli* to macerate plant tissue and display soft rot-like symptoms (7). Highly purified pectate lyase preparations macerate plant tissue, thus confirming a causal relationship between the enzymes and soft rot diseases (8).

Pectate lyases constitute a family of isozymes that share 29–91% sequence similarity (9). All isozymes cleave 1,4-linked galacturonic acid units of the pectate component of the plant cell wall by a \(\beta\)-elimination mechanism. In vitro catalyzed reactions have a bell-shaped pH profile, which extends from 6 to 10 with a pH optimum near 9.0 for most isozymes (10). Pectate lyases require Ca\(^{2+}\) for in vitro activity and presumably utilize the abundant Ca\(^{2+}\) in the plant cell wall for in vivo activity (11). Although Ca\(^{2+}\) once was assumed to bind only the substrate (12), now it is known that Ca\(^{2+}\) binds directly to the protein. Several three-dimensional structures of pectate lyases have been determined in the presence and absence of Ca\(^{2+}\) (13–16). All such structures reveal a novel \(\beta\)-helical topology that probably is shared by all family members. The structures of *Bacillus subtilis* pectate lyase (15) and *Bacillus* sp. strain KSM-P15 pectate lyase (16) reveal details of a Ca\(^{2+}\)-binding site at the same relative location in each structure. Another structure, that of a complex between an inactive PelC1 mutant and a pentagalacturonate substrate, has revealed three additional Ca\(^{2+}\) sites linking the substrate to the protein (17). Although the PelC R218K-(Ca\(^{2+}\))₄-pentaGalA structure provides many insights into the pectate lyase cleavage mechanism, the structure complicates questions regarding the function of the Ca\(^{2+}\) ions. Are all Ca\(^{2+}\) ions essential for in vitro catalytic activity? Are the Ca\(^{2+}\) ions only required for substrate binding, or do any have an active catalytic role? If so, which Ca\(^{2+}\) ion has an active catalytic role, and how might the ion participate in catalysis? Is the primary Ca\(^{2+}\) ion involved in the polygalacturonic acid hydrolyase activity that is observed in some pectate lyases (18, 19)? In view of the observations that the pH of the plant cell wall increases as the pectate component is degraded (20), how does pH affect the affinity of the Ca\(^{2+}\) ions for PelC? To address these questions, various studies with PelC have been initiated. The results presented herein explore the binding of the primary Ca\(^{2+}\) ion to PelC over a broad pH range.

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1 The abbreviations used are: PelC, *Erwinia chrysanthemi* pectate lyase C; GalA, galacturonic acid; bis-tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.
EXPERIMENTAL PROCEDURES

Crystal Preparation—PelC was purified and crystallized from ammonium sulfate according to published protocols (21). Unless noted, all reagents were purchased from Sigma. The PelC-Ca\(^{2+}\) complexes were prepared by a gradual reduction in ammonium sulfate and a concurrent increase in polyethylene glycol 8000 surrounding the native crystals. Subsequently, Ca\(^{2+}\) was added gradually. The crystals were sensitive to the order and rate of the changes made to the mother liquor, as illustrated in Fig. 1. PelC crystals were prepared at three pH values: 4.5, 9.5, and 11.2. At pH 4.5 and 11.2, the reactions were buffered by 100 mM \(\gamma\)-aminobutyric acid. At pH 9.5, 100 mM amino-2-methyl-1,3-propanediol buffer was used. At each pH, crystals were prepared in the absence of Ca\(^{2+}\) or at Ca\(^{2+}\) concentrations of 1, 5, 10, 20, or 30 mM.

Data Collection and Processing—X-ray diffraction data were collected at 20 °C using a dual multilayer area detector system (San Diego Multiwire Systems, San Diego, CA). The data were processed by the San Diego Multiwire software package (22) and placed on an absolute scale by the Wilson method (23). The x-ray data statistics are summarized in Table I.

Model Refinement—Initial phases were derived from the native PelC structure determined at pH 7.5 in the absence of Ca\(^{2+}\) (24). The reflection data were divided randomly into two sets, a working set composed of 90% of the data and a test set composed of the remaining data used for cross-validation of the refinement cycles (25). The parameter and topology files of Engh and Huber were used (26). The weight of the crystallographic term in the molecular dynamics was calculated from the empirical check procedure of X-PLOR (27) and kept constant at 50% throughout the refinement process.

The Ca\(^{2+}\) ion for each data set was confirmed by difference Fourier methods. Each model was refined by using simulated annealing methods as implemented by X-PLOR (26). Alterations within a 10 Å region around the Ca\(^{2+}\) were identified by simulated annealed OMIT electron density maps and refit manually using O (28) and OOPS (29). Following Powell minimization and temperature factor refinement, water molecules were assigned to peaks < 4σ using MAPMAN (30) and verified visually or by using reasonable distance and geometry criteria. In the final stages, alternate cycles of occupancy and temperature factor refinement of Ca\(^{2+}\) and selected atoms were carried out in X-PLOR until convergence was reached.

To estimate the error in the Ca\(^{2+}\) occupancy, each of the 11 final models was refined by energy minimization using 15 different values of Ca\(^{2+}\) occupancy ranging from 0.1 to 1.5. The total energy for each model was plotted against the occupancy of the Ca\(^{2+}\) ion, and a nonlinear regression analysis was carried out using SigmaPlot 5.0. Each plot was fitted using a four-parameter gaussian curve, and all had R\(^2\) values exceeding 0.99. The final Ca\(^{2+}\) occupancy was assumed to be the value corresponding to the energy minimum of each plot, and the estimated error for Ca\(^{2+}\) occupancy was obtained from the regression analysis. The final refinement statistics for each model are summarized in Table I.

Model Analysis—Crystallographic models were checked with PROCHECK (31) for geometric and crystallographic statistics. The superposition of a-carbons for each model was calculated and refined using least squares methods implemented by LSQMAN (32). All model figures were prepared using SETOR (33).

Estimation of \(pK_a\) Values—The \(pK_a\) values of individual amino acids were estimated using the computer program MEAD (34) and the atomic coordinates of the PelC structures at pH 9.5 in the absence and presence of 30 mM Ca\(^{2+}\). All water molecules were included in the calculations. The atoms were assigned partial charges and atomic radii using the standard CHARMM parameters (35). Interior dielectric constants of 4, 50, 30, 20, 35, or 40 were used in the calculations.

Apparent Dissociation Constant—The Ca\(^{2+}\) occupancy for each x-ray diffraction data set at a given pH was plotted using SigmaPlot 5.0. The data were fitted to a single rectangular, two-parameter hyperbolic curve using regression analysis. The apparent dissociation constant (\(K_a\)) of Ca\(^{2+}\) from PelC at each pH was extrapolated subsequently from the 50% occupancy value.

Intrinsic Tryptophan Fluorescence Assay—The apparent \(K_a\) of Ca\(^{2+}\) from PelC at pH 9.5 also was determined by changes in the intrinsic tryptophan fluorescence. The four fluorescent changes were monitored on an SPEX 112 fluorometer with a 150-watt Xenon Arc as the source at an excitation wavelength of 284 nm and over an emission wavelength range of 300–400 nm at a 2-nm interval. Water-Raman spectra were collected before each measurement for experimental consistency. The integral of each emission spectrum was used to express the fluorescence intensity of PelC at each Ca\(^{2+}\) concentration after correcting for dilution resulting from the titrant added. For the titration of PelC, an appropriate concentration of Ca\(^{2+}\) was added to a 2-mL fluorescence cuvette containing 0.088 mM PelC in 5 mM bis-tris propane, pH 9.5, to give a final concentration of Ca\(^{2+}\) in the solution ranging from 10^{-6} to 10^{-1} M. After addition of the titrant, the sample was incubated for 2 min to ensure that equilibrium had been attained before an emission spectrum was collected. Fluorescence measurements were carried out at room temperature. The apparent \(K_a\) value was defined as the Ca\(^{2+}\) concentration that elicited one-half of the maximum change in fluorescence, which was determined by curve fitting with nonlinear regression to a sigmoidal dose-response curve using Prism 3.0 (GraphPad Software, San Diego, CA).

Pectolytic Activity Assay—The optimal Ca\(^{2+}\) concentration was determined at each pH using the standard spectroscopic method for determining pectate lyase activity (8). At pH 4.5 and 11.2, the reactions were buffered with 100 mM \(\gamma\)-aminobutyric acid. At pH 9.5, 100 mM amino-2-methyl-1,3-propanediol buffer was used. The selected buffer, varying concentrations of CaCl\(_2\), and 100 µl of a 1% (w/v) sodium polypectate stock were premixed in a total volume of 990 µl. The tested Ca\(^{2+}\) concentrations ranged from 0.05 to 2 mM. To initiate the reaction, 10 µl of a PelC stock (26 nm) were added and mixed. Enzymatic activity was measured by recording the absorbance increase at 232 nm every 10 s at 22 °C. One unit of pectate lyase activity is defined as the production of 1 nmol of unsaturated product/min. The formation of 1 nmol of unsaturated uronide/min was taken to correspond to 1.73 units/min (36).

RESULTS

Structural Models—11 structural models of PelC have been constructed and refined using the x-ray diffraction data. Two PelC models have been refined in the absence of Ca\(^{2+}\) at pH 4.5 and 9.5. The root mean square deviation between the a-carbons of the two models is 0.13 Å, indicating insignificant changes in the PelC structure over a broad pH range. Additional structures of PelC have been refined in the presence of 5, 20, and 30 mM Ca\(^{2+}\) at pH 4.5, in the presence of 0.3, 5, and 30 mM Ca\(^{2+}\) at pH 9.5, and in the presence of 1, 5, and 30 mM Ca\(^{2+}\) at pH 11.2. In these models, the Ca\(^{2+}\) occupancy ranges from 38 to 92% with the highest occupancy found at the 30 mM Ca\(^{2+}\) concentration, pH 9.5. There are only two significant structural changes coinciding with the presence or absence of Ca\(^{2+}\). Major alterations are observed in the conformation of Asp-129 and in the occupancy of four water molecules near the Ca\(^{2+}\) binding site. Consequently, in those models with a partial Ca\(^{2+}\) occupancy, the occupancy of each Asp-129 conformation and the occupancies of the water molecules also have been refined. Essentially, each refined model is a composite of two partial models, one representing the PelC structure in the absence of Ca\(^{2+}\) and the other representing the structure in the presence of Ca\(^{2+}\). The refinement statistics for all models are summarized in Table I.

Details of the Ca\(^{2+}\) Site—The description of the Ca\(^{2+}\) site is based on atomic details obtained from the Pel-Ca\(^{2+}\) structure...
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**Table I**

Crystallographic data and refinement statistics

| pH          | 11.2 | 11.2 | 11.2 | 9.5 | 9.5 | 9.5 | 9.5 | 4.5 | 4.5 | 4.5 | 4.5 | 4.5 |
|-------------|------|------|------|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Ca\(^{2+}\) (mm) | 30.0 | 5.0  | 1.0  | 30.0| 5.0 | 0.3 | 0.0 | 30.0| 30.0| 30.0| 5.0 | 0.0 |
| Space group  | P2\(_1\)2\(_1\)2\(_1\) | P2\(_1\)2\(_1\)2\(_1\) | P2\(_1\)2\(_1\)2\(_1\) | P2\(_1\)2\(_1\)2\(_1\) | P2\(_1\)2\(_1\)2\(_1\) | P2\(_1\)2\(_1\)2\(_1\) | P2\(_1\)2\(_1\)2\(_1\) | P2\(_1\)2\(_1\)2\(_1\) | P2\(_1\)2\(_1\)2\(_1\) | P2\(_1\)2\(_1\)2\(_1\) | P2\(_1\)2\(_1\)2\(_1\) | P2\(_1\)2\(_1\)2\(_1\) |
| a (Å)       | 72.82 | 72.67 | 71.48 | 72.71 | 73.18 | 71.99 | 73.32 | 72.89 | 71.91 | 71.77 | 72.94 |
| b (Å)       | 81.07 | 80.98 | 76.76 | 80.80 | 81.06 | 78.27 | 80.93 | 80.93 | 80.99 | 81.02 | 80.75 |
| c (Å)       | 95.77 | 95.37 | 91.67 | 95.74 | 94.93 | 95.78 | 95.02 | 95.83 | 95.94 | 95.78 | 95.77 |
| Resolution (Å) | 2.20 | 2.20 | 2.20 | 2.20 | 2.20 | 2.20 | 2.20 | 2.20 | 2.20 | 2.20 | 2.20 |
| Total observations | 92,542 | 134,895 | 99,422 | 178,814 | 128,630 | 108,490 | 115,096 | 353,660 | 171,235 | 136,285 | 133,466 |
| Multiplicity | 2.87 | 2.84 | 4.10 | 4.18 | 4.05 | 3.80 | 4.11 | 7.48 | 4.42 | 4.72 | 3.45 |
| % completeness | 94.11 | 99.68 | 92.30 | 99.78 | 89.18 | 99.95 | 98.84 | 99.95 | 99.88 | 82.02 | 99.44 |
| Average I/\(I_0\) | 9.99 | 8.77 | 9.60 | 7.79 | 6.76 | 12.70 | 10.42 | 9.29 | 12.31 | 11.17 | 10.40 |
| \(R_{sym}\) | 4.22 | 7.67 | 7.60 | 6.34 | 7.93 | 4.20 | 5.80 | 6.81 | 4.91 | 5.51 | 4.81 |
| Protein atoms | 2649 | 2649 | 2649 | 2649 | 2649 | 2649 | 2649 | 2649 | 2649 | 2649 | 2649 |
| Waters | 162 | 145 | 191 | 150 | 145 | 189 | 170 | 170 | 177 | 139 | 168 |
| \(R_{free}\) | 0.1691 | 0.1807 | 0.2122 | 0.1841 | 0.1760 | 0.2061 | 0.1822 | 0.1758 | 0.1807 | 0.1719 | 0.1767 |
| \(R_{work}\) | 0.1691 | 0.1807 | 0.2122 | 0.1841 | 0.1760 | 0.2061 | 0.1822 | 0.1758 | 0.1807 | 0.1719 | 0.1767 |

*\(R_{sym}\) = 100 \times \Sigma |I_{avg} - I_{obs}| / |I_{avg}|, where \(I_{sym}\) is the average intensity and \(I_{obs}\) is the observed intensity of the reflection.

*\(R_{free}\) = \(\Sigma F_{o} - |F_{o}|/|F_{o}|\) for the 10% of the reflections that were set aside for cross-validation and not used in the refinement. \(F_{o}\) is the observed structure factor amplitude, and \(F_{e}\) is the calculated structure factor amplitude.

*\(R_{work}\) = \(\Sigma F_{o}/\Sigma F_{e}\) for the 90% of the reflections that were set aside for cross-validation and not used in the refinement.

with the highest Ca\(^{2+}\) occupancy; this is the model at pH 9.5 and 30 mM Ca\(^{2+}\). The details of the Ca\(^{2+}\) site are compared with the same region in the absence of Ca\(^{2+}\) (Fig. 2). The Ca\(^{2+}\) site is located at the same position as the Lu\(^{3+}\) derivative of PelC (13) and is similar to the Ca\(^{2+}\) site observed in B. subtilis pectate lyase (15). Seven oxygen atoms form, the standard bipentagonal arrangement around the Ca\(^{2+}\) ion with an average Ca\(^{2+}\)-oxygen bond distance of 2.4 Å. The individual Ca\(^{2+}\)-oxygen bond distances are listed in Table II. Two of the coordinated oxygen atoms derive from water molecules, Wat-1 and Wat-2. Five oxygen atoms originate from amino acid side chains. Each oxygen of the carboxylic group of Asp-129 is coordinated. Asp-131 is in an alternate conformation. Nevertheless, the movement of Glu-166 is critical for the establishment of an extensive network of hydrogen bonds and electrostatic interactions in the active site region. In the presence of Ca\(^{2+}\), Glu-166 is locked into a singular position by the coordination of one carboxylic oxygen to Ca\(^{2+}\) and the other oxygen, with a strong electrostatic interaction with Lys-190 at a distance of 2.7 Å. In the absence of Ca\(^{2+}\), Glu-166 does not form strong interactions with neighboring atoms.

Changes in the water structure also are observed. In the absence of Ca\(^{2+}\), there are four water molecules in the region, Wat-1, Wat-2, Wat-3, and Wat-4. The position and occupancy of Wat-1 and Wat-4 are independent of the presence or absence of Ca\(^{2+}\). Wat-1 coordinates to Ca\(^{2+}\) when the ion is present but does not alter its position when the cation is absent. Similarly, Wat-4 forms a hydrogen bond with the guanidinium nitrogen of Arg-218 irrespective of the Ca\(^{2+}\) occupancy. Wat-2 forms hy-

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Fig. 2. Comparison of the structural changes in PelC with and without Ca\(^{2+}\). The backbone is represented by a green ribbon, the Ca\(^{2+}\) ion by a large yellow sphere, water molecules by small spheres, and amino acid side chains by rods. For the PelC-Ca\(^{2+}\) complex, the carbon atoms are light green, the oxygen atoms are red, and the nitrogen atoms are blue. For the Ca\(^{2+}\)-free PelC structure, the amino acids and the water molecules are shown in cyan. Asp-129 and Glu-166 are the only amino acids that undergo a conformational change upon Ca\(^{2+}\) binding. W-1, Wat-1, W-2, Wat-2, W-3, Wat-3, W-4, Wat-4, W-5, Wat-5.
dorgen bonds with Glu-166 and Asp-170 in the absence of Ca\(^{2+}\) and essentially occupies the Ca\(^{2+}\) binding site. In the presence of Ca\(^{2+}\), Wat-2 moves to a position that allows it to coordinate with the Ca\(^{2+}\) ion. Most significantly, Wat-2 forms a hydrogen-bonding network to Wat-4 via a new water molecule, Wat-5, only in the presence of Ca\(^{2+}\). This hydrogen-bonding network effectively links the Ca\(^{2+}\) ion with the catalytic amino acid Arg-218. A comparable hydrogen-bonding network, linking Arg-218 to any other atom, does not exist in the absence of Ca\(^{2+}\). Wat-3 also shifts position depending upon the Ca\(^{2+}\) occupancy. Wat-3 is displaced by the carboxylic oxygen of Asp-129 when the residue rotates into the coordination sphere of the Ca\(^{2+}\) ion. Finally, two new water molecules, Wat-6 and Wat-7, appear, forming hydrogen bonds with water molecules in the region. Overall, there is a net gain of two water molecules within an 8-Å sphere around the Ca\(^{2+}\).

**Effect of Ca\(^{2+}\) on the pK\(_a\) of Arg-218**—In the pectate lyase superfamily, an invariant arginine analogous to Arg-218 in PelC is believed to initiate the abstraction of the proton in the initial stages of the β-elimination reaction. The pK\(_a\) of Arg-218 in the PelC structures at pH 9.5, in the presence (30 mM) and absence of Ca\(^{2+}\), has been estimated by macroscopic electrostatic calculations using different values of the dielectric constant. Using a dielectric constant of 20, the pK\(_a\) of Arg-218 is estimated to be 13.0 in the absence of Ca\(^{2+}\) and 11.60 in the presence of Ca\(^{2+}\). Although the value of the dielectric constant influences the absolute pK\(_a\) value, the relative shifts in the pK\(_a\)s are qualitatively similar. At all dielectric constant values, the presence of Ca\(^{2+}\) lowers the pK\(_a\) of Arg-218, suggesting that the primary Ca\(^{2+}\) ion plays a role in priming the catalytic arginine for the proton abstraction step.

**PelC Affinity for Ca\(^{2+}\)**—The Ca\(^{2+}\) occupancy in each of the PelC-Ca\(^{2+}\) complexes shown in Table III has been plotted as a function of Ca\(^{2+}\) concentration at each pH value. The occupancy data have been fit to a single rectangular, two-parameter hyperbolic curve using regression analysis. The Ca\(^{2+}\) occupancy in each of the PelC-Ca\(^{2+}\) complexes is estimated to be 0.132 (±0.004) mM at pH 9.5, 1.09 (±0.29) mM at pH 11.2, and 5.84 (±0.41) mM at pH 4.5 (Fig. 3). The apparent K\(_d\) of the PelC-Ca\(^{2+}\) complex is estimated to be 0.132 (±0.004) mM at pH 9.5, 1.09 (±0.29) mM at pH 11.2, and 5.84 (±0.41) mM at pH 4.5 (Fig. 3). The apparent K\(_d\) at pH 9.5 has been verified by intrinsic tryptophan fluorescence techniques. The addition of Ca\(^{2+}\) to native PelC induces a small but measurable change in the intensity of the emitted fluorescence but does not shift the wavelength of the emitted light. The apparent K\(_d\) from the fluorescence data is estimated from the titration curve (Fig. 4). At pH 9.5, the K\(_d\) of 0.133 (±0.045) mM estimated from fluorescence methods is in close agreement with a K\(_d\) of 0.132 (±0.004) mM measured from the x-ray diffraction analyses of the Ca\(^{2+}\) occupancy. At all pH values, the PelC affinity for Ca\(^{2+}\) is relatively weak.

**OPTIMAL Ca\(^{2+}\) CONCENTRATION FOR ENZYMATIC ACTIVITY**—The pH profiles of all pectate lyases are determined routinely at Ca\(^{2+}\) concentrations between 0.1 and 0.4 mM. Because the PelC affinity for Ca\(^{2+}\) at pH 4.5 is weak, a higher concentration of Ca\(^{2+}\) may be needed to activate PelC at the lower pH range. The enzymatic activity of PelC at pH 4.5 has been determined in the presence of various Ca\(^{2+}\) concentrations ranging from 2.5 to 40 mM. The data demonstrate that the enzymatic activity at pH 4.5 is greatest at 30 mM Ca\(^{2+}\) (Fig. 5). Saturation of the Ca\(^{2+}\) binding site increases the enzymatic activity by 10-fold. Nevertheless, at pH 4.5, PelC still has very low activity, less than 1% of the maximal activity measured at pH 9.5.

**DISCUSSION**

The primary Ca\(^{2+}\) site of PelC shares the same location and coordination as the primary Ca\(^{2+}\) site for the heavy atom derivative Lu\(^{3+}\), which is used to phase the initial structure. The Ca\(^{2+}\) coordination involves three amino acids and two water molecules arranged in the typical bipentagonal coordination. The primary Ca\(^{2+}\) ion has been observed in two other pectate lyase structures, that of *B. subtilis* pectate lyase (15) and Bacillus sp. strain KSM-P15 pectate lyase (16). Although the location and coordination numbers are the same, there are small differences between the Ca\(^{2+}\) binding sites. Asp-129 in PelC corresponds to Gln-182 in *B. subtilis* pectate lyase. As an uncharged residue, Gln-182 is unable to coordinate to the Ca\(^{2+}\).

![Graph showing Ca\(^{2+}\) occupancy in PelC](image)

**TABLE II**

| Oxygen atom | Distance |
|-------------|----------|
| Asp-129 O11 | 2.39Å    |
| Asp-131 O11 | 2.59Å    |
| Asp-131 O22 | 2.47Å    |
| Glu-166 O1  | 2.39Å    |
| Asp-170 O2  | 2.41Å    |
| Wat-1 O1   | 2.45Å    |
| Wat-2 O1   | 2.50Å    |
| **Average** | 2.46Å    |

**TABLE III**

| Oxygen values of Asp-129 and Ca\(^{2+}\) | pH 11.2 | pH 11.2 | pH 11.2 | pH 9.5 | pH 9.5 | pH 9.5 | pH 4.5 | pH 4.5 | pH 4.5 |
|---------------------------------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
|                                       | (30 mM Ca\(^{2+}\)) | (5 mM Ca\(^{2+}\)) | (1 mM Ca\(^{2+}\)) | (30 mM Ca\(^{2+}\)) | (5 mM Ca\(^{2+}\)) | (0.5 mM Ca\(^{2+}\)) | (30 mM Ca\(^{2+}\)) | (20 mM Ca\(^{2+}\)) | (5 mM Ca\(^{2+}\)) |
| Ca\(^{2+}\) (%)                      | 87.7 ± 0.3 | 83.5 ± 0.1 | 42.3 ± 2.5 | 92.1 ± 0.4 | 89.2 ± 0.4 | 64.0 ± 0.2 | 68.5 ± 0.2 | 65.0 ± 0.3 | 37.9 ± 1.7 |
| Asp-129a\(^\text{a}\)                | 82     | 70     | 40     | 86     | 85     | 45     | 67     | 48     | 41     |
| Asp-129b\(^\text{b}\)                | <15    | <15    | 30     | <15    | <15    | 60     | <15    | 28     | 26     |

\(^\text{a}\) Asp-129a is the conformation of Asp-129 in the presence of Ca\(^{2+}\).

\(^\text{b}\) Asp-129b is the conformation of Asp-129 in the absence of Ca\(^{2+}\).
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A

![Graph A](image)

**Fig. 4.** The effect of Ca\textsuperscript{2+} titration upon the intrinsic tryptophan fluorescence of PelC. A, the emission spectra of the intrinsic tryptophan fluorescence of PelC is shown as a function of wavelength. A small but reproducible change in the intensity but not the wavelength of the emission spectrum of PelC is observed when Ca\textsuperscript{2+} is added. When Ca\textsuperscript{2+} is chelated by the addition of EGTA, the spectral changes return to base line. For clarity, the spectra for Ca\textsuperscript{2+} concentrations between 0 and 5 mM are not shown. ———, PelC in the absence of Ca\textsuperscript{2+}; ———, PelC in the presence of 5 mM Ca\textsuperscript{2+} and 5 mM EGTA; ———, PelC in the presence of 5 mM Ca\textsuperscript{2+}. B, the difference between the fluorescence of PelC in the absence of Ca\textsuperscript{2+} (F\textsubscript{0}) and in the presence of Ca\textsuperscript{2+} (F) is plotted as a function of the Ca\textsuperscript{2+} concentration. The titration of PelC with Ca\textsuperscript{2+} has been carried out in 5 mM bis-tris propane at a pH of 9.5 as described under “Experimental Procedures.” The error bars have been calculated from 14 measurements.

B

![Graph B](image)

The effect of Ca\textsuperscript{2+} titration upon the intrinsic tryptophan fluorescence of PelC. A, the emission spectra of the intrinsic tryptophan fluorescence of PelC is shown as a function of wavelength. A small but reproducible change in the intensity but not the wavelength of the emission spectrum of PelC is observed when Ca\textsuperscript{2+} is added. When Ca\textsuperscript{2+} is chelated by the addition of EGTA, the spectral changes return to base line. For clarity, the spectra for Ca\textsuperscript{2+} concentrations between 0 and 5 mM are not shown. ———, PelC in the absence of Ca\textsuperscript{2+}; ———, PelC in the presence of 5 mM Ca\textsuperscript{2+} and 5 mM EGTA; ———, PelC in the presence of 5 mM Ca\textsuperscript{2+}. B, the difference between the fluorescence of PelC in the absence of Ca\textsuperscript{2+} (F\textsubscript{0}) and in the presence of Ca\textsuperscript{2+} (F) is plotted as a function of the Ca\textsuperscript{2+} concentration. The titration of PelC with Ca\textsuperscript{2+} has been carried out in 5 mM bis-tris propane at a pH of 9.5 as described under “Experimental Procedures.” The error bars have been calculated from 14 measurements.

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between 10 and 50 mM (39). The x-ray diffraction method is somewhat unconventional for measuring Ca\textsuperscript{2+} affinity, but it has been used previously in the quantitation of Ca\textsuperscript{2+} binding to internalin B (40). Moreover, the K\textsubscript{d} at pH 9.5 determined by the x-ray diffraction method has been corroborated with intrinsic tryptophan fluorescence measurements. The present research does not address whether the affinity of Ca\textsuperscript{2+} for PelC increases in the presence of the substrate. Such a question is difficult to answer because additional Ca\textsuperscript{2+} ions are bound at the PelC-oligosaccharide interface (17).

Although the x-ray diffraction method has many drawbacks over conventional methods for determining Ca\textsuperscript{2+} affinity for a protein, the approach offers one significant advantage in that the actual Ca\textsuperscript{2+} ion for which K\textsubscript{d} is being measured can be visualized. The diffraction studies of the PelC R218-(Ca\textsuperscript{2+})\textsubscript{4}-pentaGalA complex as well as preliminary fluorescence data indicating a negative Hill coefficient raised the possibility that native PelC might have more than one Ca\textsuperscript{2+} site, each with differing affinities, at exogenous Ca\textsuperscript{2+} concentrations of 10 mM or less. No positive electron density, consistent with partially occupied Ca\textsuperscript{2+} ions, is present at any other site at which the exogenous Ca\textsuperscript{2+} concentration is 30 mM or less. This observation is consistent with the R218-(Ca\textsuperscript{2+})\textsubscript{4}-pentaGalA complex structure in which the other Ca\textsuperscript{2+} ions coordinate to only one or two amino acids, suggesting that the affinity of these Ca\textsuperscript{2+} ions for the protein may be very weak (17).

The Ca\textsuperscript{2+} affinity for PelC is highest at pH 9.5, the optimal pH for *in vitro* enzymatic activity. The Ca\textsuperscript{2+} affinity decreases 10-fold at pH 11.2, possibly as a consequence of the affinity of Ca\textsuperscript{2+} for water. At pH 11.2, there is a large population of hydroxyl ions that will compete effectively with the amino acids for direct coordination to Ca\textsuperscript{2+}. The nearly 100-fold reduction in Ca\textsuperscript{2+} affinity for PelC at pH 4.5 can be explained by the increased protonation of the aspartic and glutamic acid groups that otherwise would coordinate with the Ca\textsuperscript{2+} ion. The detection of any Ca\textsuperscript{2+} affinity at pH 4.5 is somewhat surprising, given that *in vitro* pectate lyase activity had not been detected previously under standard assay conditions. However, the standard assays routinely employ Ca\textsuperscript{2+} concentrations that are too low at pH 4.5 to saturate the Ca\textsuperscript{2+} site as deduced by the x-ray diffraction analyses. This observation raises the question as to what extent pectate lyase activity at pH 4.5 is dependent upon the Ca\textsuperscript{2+} concentration. To answer this question, we have determined pectate lyase activity in the presence of higher Ca\textsuperscript{2+} concentrations and found that saturation of the Ca\textsuperscript{2+} binding site increases the enzymatic activity by 10-fold. Nevertheless, the activity of PelC at pH 4.5 still is very low, as it is
less than 1% of the maximal activity measured at pH 9.5. Therefore, the low pectate lyase activity at pH 4.5 must be dependent upon additional factors.

The Ca$^{2+}$ binding motif found in PelC differs from the high affinity Ca$^{2+}$-sites found in EF-hand Ca$^{2+}$-binding proteins (41–45). The typical EF-hand Ca$^{2+}$-binding site consists of a continuous or discontinuous helix-loop-helix motif on which the coordinating amino acids are located. In the continuous type (Fig. 6A), the helix-loop-helix motif is composed of a contiguous sequence of 12 amino acids. In the discontinuous type (Fig. 6B), the helix-loop-helix is composed of two noncontiguous sequences of amino acids. In contrast to the typical EF-hand Ca$^{2+}$-binding motif, the Ca$^{2+}$-binding pocket on PelC is composed entirely of β-strands and β-turns (Fig. 6C). The coordinating amino acids are located on two adjacent runs of the parallel β-helix. Three of the four amino acids that coordinate the Ca$^{2+}$ ion are located on β-turns that connect two β-strands. Only one amino acid, Asp-170, is located within a β-strand. Despite the differences in the surrounding topology of the Ca$^{2+}$ sites, the coordination around the Ca$^{2+}$ ion in PelC appears to be identical in number and average bond distance to typical helix-loop-helix proteins that have much higher Ca$^{2+}$ affinities.

Why then does PelC bind Ca$^{2+}$ weakly, whereas proteins with a helix-loop-helix motif bind Ca$^{2+}$ with high affinity? In reviews of Ca$^{2+}$-binding proteins (44–46), no one has been able to find a direct correlation between the metal ion binding site and its affinity for the protein. Pidcock and Moore (46) observed other features that may affect the strength of binding. Among these features are the nature of the electrostatic interactions, including extensive hydrogen bond networks, and large metal ion-induced protein conformational changes. As discussed previously, PelC has an extensive hydrogen bond network that is altered in the presence of Ca$^{2+}$. The Ca$^{2+}$-induced conformational changes are relatively minor in PelC and less likely to influence the affinity of Ca$^{2+}$. We propose that the answer for PelC may lie in the orientation of the Ca$^{2+}$ toward the lone pairs of electrons on the carboxylate oxygen. High affinity for a Ca$^{2+}$ ion usually is found when Ca$^{2+}$ is coordinated directly to both oxygen atoms or to the syn conformation of the lone electron pair on one of the carboxylate oxygens (47). In PelC, the carboxylate oxygen atoms of two amino acids, Glu-166 and Asp-170, coordinate to Ca$^{2+}$ in the anti conformation of the lone electron pair. Asp-131 forms a direct interaction with the Ca$^{2+}$, and only Asp-129 binds in the syn conformation. Thus one possible explanation for the weaker Ca$^{2+}$ affinity in PelC may be the weak coordination to the anti conformation in two of the four coordinating amino acids.

In the presence of a substrate, PelC is known to bind to three additional Ca$^{2+}$ ions, each of which has fewer coordinating ligands to the protein. None of these Ca$^{2+}$ ions are observed in the PelC structure in the absence of a substrate. The presence of only one Ca$^{2+}$, which coordinates more tightly than the other sites through an invariant and several highly conserved amino acids, suggests that this Ca$^{2+}$ has a special role. One role, suggested by the present study, is to reduce the pK$_a$ of Arg-218 so that the amino acid is better suited chemically to act as a proton acceptor in the β-elimination reaction mechanism. Another possibility, suggested by the PelC R218R-(Ca$^{2+}$)$_3$-penta-GalP complex, is that this Ca$^{2+}$ is critical for aligning the substrate properly in the active site. Other studies suggest that the occupancy of the primary Ca$^{2+}$ site determines whether PelC functions as a hydrolase or a lyase (48). When the structure of the PelC-Ca$^{2+}$ complex is superimposed upon the polygalacturonase structures (48), the PelC amino acids coordinated to the primary Ca$^{2+}$ ion are analogous to the catalytic residues in polygalacturonases. The PelC amino acids are in the proper orientation to carry out hydrolase activity but only in the absence of the Ca$^{2+}$ ion. Thus the weak affinity of PelC for the primary Ca$^{2+}$ ion, as determined herein, is critical for understanding how Ca$^{2+}$ can regulate the function of PelC.

Although structure-function studies have focused primarily on microbial pectate lyases, pel genes have been identified in a wide range of plants with at least 25 isozymes found by gene annotation in *Arabidopsis thaliana* (49). Because the plant cell wall pH is ~5.75, it has been difficult to understand how any pectate lyase functions in such an environment. The present study indicates that pectate lyases are active even at a low pH if the exogenous Ca$^{2+}$ concentration is sufficiently high. If the Ca$^{2+}$ concentration is low, other studies suggest that the enzyme function as hydrolases. The occupancy of the primary Ca$^{2+}$ site appears to determine whether the pectate lyase functions as a hydrolase or a lyase. Although additional studies are needed to confirm this hypothesis, the weak affinity of the primary Ca$^{2+}$ ion for PelC observed herein is a necessary feature for the Ca$^{2+}$ ion to serve as a regulatory switch.

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2 P. M. Coutinho and B. Henrissat, carbohydrate-active enzymes server at afmb.cnrs-mrs.fr/~cazy/CAZYindex.html.
Ca$^{2+}$ Binding to Pectate Lyase C

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REFERENCES

1. Barras, F., Van Gijsegem, F., and Chatterjee, A. K. (1994) Annu. Rev. Phytopathol. 32, 291–234

2. Collmer, A., and Keen, N. T. (1986) Annu. Rev. Phytopathol. 24, 383–409

3. Boccara, M., and Chatan, V. (1989) J. Bacteriol. 171, 4085–4087

4. Boccara, M., Dielez, A., Reuve, M., and Kotoujansky, A. (1988) Physiol. Mol. Plant Pathol. 33, 95–104

5. Ried, J. L., and Collmer, A. (1988) Mol. Plant-Microbe Interact. 1, 22–38

6. Thurn, K. J., and Chatterjee, A. K. (1985) Appl. Environ. Microb. 50, 894–898

7. Tamaki, S. J., Gold, S., Robeson, M., Manulis, S., and Keen, N. T. (1988) J. Bacteriol. 170, 3468–3478

8. Kita, N., Boyd, C. M., Garrett, M. R., Jurnak, F., and Keen, N. T. (1996) J. Biol. Chem. 271, 26529–26535

9. Heffron, S., Henriussat, B., Yoder, M. D., Lietzke, S., and Jurnak, F. (1995) Mol. Plant-Microbe Interact. 8, 331–334

10. Tardy, F., Nasser, W., Robert-Baudouy, J., and Hugouvieux-Cotte-Pattat, N. (1997) J. Bacteriol. 179, 2503–2511

11. Hepler, P. K., and Wayne, R. O. (1985) Annu. Rev. Plant Physiol. 36, 397–439

12. Crawford, M. S., and Kolattukudy, P. E. (1987) Arch. Biochem. Biophys. 258, 196–205

13. Yoder, M. D., and Jurnak, F. (1995) Plant Physiol. 107, 349–364

14. Lietzke, S. E., Keen, N. T., Yoder, M. D., and Jurnak, F. (1994) Plant Physiol. 106, 849–862

15. Pickrell, R. J., Jenkins, J., Harris, G., Nasser, W., and Robert-Baudouy, J. (1994) Nat. Struct. Biol. 1, 717–723

16. Akita, M., Suzuki, A., Kobayashi, T., Ito, S., and Yamane, T. (2001) Acta Crystallogr. Sect. D Biol. Crystallogr. 57, 1786–1792

17. Scavetta, R. D., Herron, S. R., Hotchkiss, A. T., Kita, N., Keen, N. T., Benen, J. A. E., Rester, H. C. M., Visser, J., and Jurnak, F. (1999) Plant Cell 11, 1081–1092

18. Hotchkiss, A. T., Jr., and Hicks, K. B. (1993) Carbohydr. Res. 247, 1–7

19. Hotchkiss, A. T., Jr., Revear, L. G., and Hicks, K. B. (1996) Physiol. Mol. Plant Pathol. 48, 1–9

20. De Lorenzo, G., Cervone, F., Hahn, M. G., Darvill, A., and Albersheim, P. (1994) Physiol. Mol. Plant Pathol. 39, 335–344

21. Yoder, M. D., DeChaine, D. A., and Jurnak, F. (1990) J. Biol. Chem. 265, 11429–11433

22. Howard, A. J., Nielson, C., and Young, N. H. (1985) Methods Enzymol. 114, 452–472

23. Wilson, A. C. (1942) Nature 150, 90–112

24. Yoder, M. D., Keen, N. T., and Jurnak, F. (1993) Science 260, 1503–1507

25. Brunger, A. T. (1993) Acta Crystallogr. Sect. D Biol. Crystallogr. 49, 24–36

26. Engh, R., and Huber, R. (1991) Acta Crystallogr. Sect. A 47, 392–400

27. Brunger, A. T. (1996) X-FLOR. Version 3.8, Yale University, New Haven, CT

28. Jones, T. A., Zou, J. Y., Cowan, S. W., and Kjeldgaard, M. (1991) Acta Crystallogr. Sect. A 47, 110–119

29. Kleywegt, G. J., and Jones, T. A. (1996) Acta Crystallogr. Sect. D Biol. Crystallogr. 52, 829–832

30. Kleywegt, G. J., and Jones, T. A. (1996) Acta Crystallogr. Sect. D Biol. Crystallogr. 52, 826–828

31. Laskowski, R. A., MacArthur, M. W., Moss, D. S., and Thornton, J. M. (1992) J. Appl. Crystallogr. 26, 283–291

32. Kleywegt, G. J., and Jones, T. A. (1995) Structure 3, 535–540

33. Evans, S. V. (1993) J. Mol. Graph. 11, 134–138

34. Lim, C., Bashford, D., and Karplus, M. (1991) J. Phys. Chem. 95, 5619–5620

35. Brooks, B. R., Bruccoleri, R. E., Olafson, B. D., and States, D. J. (1983) J. Comput. Chem. 4, 187–217

36. Zuber, M., and Hankin, L. (1970) J. Bacteriol. 104, 13–18

37. Henriussat, B., Heffron, S. E., Yoder, M. D., Lietzke, S. E., and Jurnak, F. (1995) Plant Physiol. 107, 963–976

38. Riouoy, C., Jauneau, A., Cabin-Flaman, A., Demarty, M., Lefèvre, F., and Morvan, C. (1995) Plant Physiol. Biochem. 33, 497–508

39. Vink, S. S., and Cleland, R. E. (1988) Planta 176, 60–67

40. Marino, M., Braun, L., Cossart, P., and Ghosh, P. (1999) Mol. Cell 4, 1063–1072

41. Yap, K. L., Ames, J. B., Swindells, M. B., and Ikura, M. (1999) Proteins Struct. Funct. Genet. 37, 499–507

42. Kawasaki, H., Nakayama, S., and Kretsinger, R. H. (1998) Biometals 11, 277–285

43. Nelsen, M. R., and Chazin, W. J. (1998) Biometals 11, 297–318

44. Linse, S., and Forsén, S. (1995) in Calcium Regulation of Cellular Function (Means, A. B., ed.) pp. 89–151, Raven Press, Ltd., New York

45. Falke, J. J., Drake, S. K., Hazard, A. L., and Peersen, O. B. (1994) Q. Rev. Biophys. 27, 219–290

46. Pidcock, E., and Moore, G. R. (2001) Acta Crystallogr. Sect. D Biol. Crystallogr. 57, 1003–1008

47. Carrell, c. J., Carrell, H. L., Erlebacher, J., and Glusker, J. P. (1988) J. Am. Chem. Soc. 110, 8651–8656

48. Herron, S. R., and Jurnak, F. (2002) in Pectin and Pectinases (Schols, H., ed) Kluwer Academic Publishers, Dordrecht, The Netherlands, in press