Drp35 is a protein induced by cell wall-affecting antibiotics or detergents; it possesses calcium-dependent lactonase activity. To determine the molecular basis of the lactonase activity, we first solved the crystal structures of Drp35 with and without Ca$^{2+}$; these showed that the molecule has a six-bladed $\beta$-propeller structure with two calcium ions bound at the center of the $\beta$-propeller and surface region. Mutational analyses of evolutionarily conserved residues revealed that the central calcium-binding site is essential for the enzymatic activity of Drp35. Substitution of some other amino acid residues for the calcium-binding residues demonstrated the critical contributions of Glu48, Asp138, and Asp236 to the enzymatic activity. Calcium-binding residues demonstrated the critical contributions of Glu48, Asp138, and Asp236 to the enzymatic activity. Differential scanning calorimetric analysis revealed that the loss of activity of E48Q and D236N, but not D138N, was attributed to their inability to hold the calcium ion. Further structural analysis of the D138N mutant indicates that it lacks a water molecule bound to the calcium ion rather than the calcium ion itself. Based on these observations and structural information, a possible catalytic mechanism in which the calcium ion and its binding residues play direct roles was proposed for the lactonase activity of Drp35.

Staphylococcus aureus is a major cause of hospital- and community-acquired infections. S. aureus causes serious and fatal diseases, such as toxic shock syndrome or septicemia (1). Moreover, S. aureus has the remarkable and unfortunate feature that it can become readily resistant to antibiotics. Indeed, it has acquired resistance to almost all antibiotics so far, resulting in an increase in incidence of acute hospital-acquired infections (2).

Extensive studies have focused on how S. aureus acquires resistance to antibiotics, and genome sequencing analysis confirmed the existence of many resistance genes acquired by horizontal transfer from other species (3). In addition, S. aureus can cope with antibiotic stresses in an adaptive manner through regulation of the expression of many genes (4).

Drp35 (a 35-kDa drug-responsive protein) is a cytoplasmic protein originally found to be markedly induced upon exposure of S. aureus to cell wall-affecting antibiotics (5). Antibiotic susceptibility experiments using a drp35 defective strain and over-expressing strain of S. aureus revealed that Drp35 is correlated with bacitracin resistance, although it did not show significant changes in minimal inhibitory concentration for $\beta$-lactams, glycopeptides, or fosfomycin (6). Drp35 can also be induced by a variety of detergents, including Nonidet P-40, Triton X-100, SDS, and CHAPS (6). These findings suggest that a broad range of stresses that perturb membrane integrity are responsible for the induction of Drp35 and that Drp35 may be a factor responsible for such general stresses rather than specific antibiotic stress.

Interestingly, Drp35 possesses calcium-dependent lactonase activity, although it has not been clarified how this activity contributes to the ability of the S. aureus cell to cope with stress (6). In eukaryotic cells, paraoxonase family proteins (PONs) act as lactonases, and these proteins also require calcium ions for their catalytic activity similarly to Drp35 (7–9). Based on these observations, it has been proposed that Drp35 is a bacterial counterpart of eukaryotic PONs (6). PONs are promiscuous enzymes and can hydrolyze not only lactone but also paraoxon, phosphotriester, and esters and thereby inactivate various organophosphates, including insecticides or nerve agents, such as sarin and soman (7, 8). Several studies also proposed a relationship of PONs to diseases, such as antiatherosclerotic activity (8, 10–12). The reaction mechanism of hydrolysis by PONs has been studied by structural analysis, directed evolution, and site-directed mutagenesis (13–15). However, their physiological role has not been determined despite many studies, including molecular characterization, of these proteins.

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The atomic coordinates and structure factors (code 2DG0, 2DG1, and 2DSO) 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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In analogy with the promiscuous function of PONs, Drp35 may play some important role in detoxification of compounds that affect the cytoplasmic membrane, and therefore, elucidating the precise function of Drp35 may provide some insight to overcome staphylococcal pathogenesis. However, Drp35 has not been characterized in detail at the molecular level, including determination of its physiological role. In the present study, we investigated its lactonase activity from a structural viewpoint. First, we report the crystal structures of Drp35 with and without calcium ions. In the presence of Ca\(^{2+}\), Drp35 has a six-bladed \(\beta\)-propeller structure with a calcium ion at its center. Comparison of lactonase activity with mutant proteins indicated the significance of residues coordinating to the calcium ion. Structural analysis of the D138N mutant and functional analyses using several mutants suggested that a water molecule coordinated to the calcium ion in the wild type may be essential for catalysis. Based on these findings, we discuss the mechanism of lactonase activity of Drp35.

**EXPERIMENTAL PROCEDURES**

**Materials**—All enzymes used in genetic engineering were obtained from Takara Shuzo (Kyoto, Japan) and Toyobo (Osaka, Japan). Isopropyl \(\beta\)-d-thiogalactopyranoside was obtained from Wako Fine Chemicals Inc. (Osaka, Japan). All other reagents were of biochemical research grade.

**Construction of Expression Vector for Drp35 and Mutant Proteins**—The gene encoding Drp35 was amplified using KOD-Plus DNA polymerase (Toyobo), with \(S. aureus\) \(\text{Mis}50\) genomic DNA as a template. The Ncol recognition sequence, CCATGG, was digested and ligation is underlined, and mutated nucleotides are indicated in italic type. The PCR products were inserted into the Ncol and BamHI sites of the pET28b vector (EMD Biosciences, San Diego, CA).

All expression vectors for mutant proteins were prepared with a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) using synthesized primers and the Drp35 expression vector described above as the template. The correctness of the DNA sequences was confirmed using an ABI 310 Genetic Analyzer (Applied Biosystems, Tokyo, Japan).

**Expression and Purification of Drp35 and Mutants**—Transformed Escherichia coli strain B834 (DE3), harboring Drp35 expression vector and pT-RIL (Stratagene, Madison, WI), was grown at 37 °C in LB medium supplemented with 50 \(\mu\)g ml\(^{-1}\) kanamycin and 34 \(\mu\)g ml\(^{-1}\) chloramphenicol until the early stationary phase. To induce expression of the desired protein, isopropyl \(\beta\)-d-thiogalactopyranoside was added to a final concentration of 0.5 \(\text{mm}\), and the culture was continued for 18 h at 25 °C. The selenomethionine derivative of Drp35 was obtained using the following primers: Drp35-S (5'-NNNNCCATGGCCATGTCAACAAAGATTTACAATTATTITATACG-3'), Drp35-AS (5'-CCGTCCTGAGTGAACCTGAAAACCTGAAAATCTTGATGACCTTTTGC-3'), Drp35-mut-S (5'-ACAGCTGAA-CCGGTTGTGAAATT-3'), and Drp35-mut-AS (5'-AAATTGCAAGCCGTTCAAGC-3') (restriction sites for digestion and ligation are underlined, and mutated nucleotides are indicated in italic type). The PCR products were inserted into the Ncol and BamHI sites of the pET28b vector (EMD Biosciences, San Diego, CA).

Cells were harvested by centrifugation at 5000 \(\times\) g for 10 min at 4 °C and then disrupted using a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan) in 50 mM Tris-HCl (pH 8.0), 300 mM NaCl. The cell debris was removed by centrifugation at 40,000 \(\times\) g for 30 min at 4 °C, and the supernatant was loaded onto a HiTrap column (GE Healthcare Biosciences AB, Uppsala, Sweden) pre-equilibrated with 50 mM Tris-HCl (pH 8.0), 300 mM NaCl. The column was washed with 50 mM Tris-HCl (pH 8.0), 300 mM NaCl, and then the adsorbed protein was eluted with 50 ml of a 0–0.5 M gradient of imidazole in 50 mM Tris-HCl (pH 8.0), 300 mM NaCl. Fractions containing Drp35 were dialyzed against 50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 1 mM EDTA and then further purified on a HiLoad 26/60 Superdex 200-70 pg column (GE Healthcare Biosciences AB) equilibrated with 50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 1 mM EDTA. Fractions containing the desired protein were collected and used for further experiments.

The Ca\(^{2+}\)-bound form of Drp35 and Drp35-D138N mutant were prepared by the same methods as described above except using the following buffers: for cell disruption and equilibration for HisTrap column, 50 mM Tris-HCl (pH 7.5), 300 mM NaCl, 1 mM CaCl\(_2\); for elution from HiTrap column, 50 mM Tris-HCl (pH 7.5), 300 mM NaCl, 1 mM CaCl\(_2\), 500 mM imidazole; for size exclusion chromatography, 50 mM Tris-HCl (pH 7.5), 300 mM NaCl, 1 mM CaCl\(_2\).

**Crystallization of Drp35, Drp35-Ca\(^{2+}\) Complex, and Drp35-D138N Mutant**—Purified Drp35 with or without CaCl\(_2\) was dialyzed against 20 mM Tris-HCl (pH 7.5) and 20 mM Tris (pH 8.0), with or without 5 mM CaCl\(_2\), respectively, and then concentrated up to 20 mg ml\(^{-1}\). Initial crystallization conditions were screened by the sparse matrix method at 20 °C, using a Crystal Screen kit, Crystal Screen 2 kit (Hampton Research, Laguna Hills, CA), Wizard I, and Wizard II (Emerald Biosstructures, Bainbridge Island, WA). Crystals of the apo form of Drp35 most suitable for further analyses were grown by the hanging drop vapor diffusion method from 100 mM Tris buffer, pH 8.8, 23% polyethylene glycol 4000, 0.1 M lithium sulfate. Crystals of Drp35 complexed with Ca\(^{2+}\) were grown from 100 mM HEPES (pH 7.6), 1050 mM succinic acid (pH 7.0), 1% polyethylene glycol monomethyl ether 2000. Crystals of the Drp35-D138N mutant were grown from a buffer containing 100 mM HEPES (pH 7.2), 1000 mM succinic acid (pH 7.0), 1% polyethylene glycol monomethyl ether 2000.

**X-ray Diffraction**—X-ray diffraction of selenomethionine-substituted Drp35 was performed on beamline NW12 at Photon Factory (Tsukuba, Japan) under cryogenic conditions (100 K). For single-wavelength anomalous diffraction (SAD) phasing, a wavelength of 0.97908 Å was chosen on the basis of the fluorescence spectrum of the selenium K absorption edge. The diffraction data of the Drp35-Ca\(^{2+}\) complex and Drp35-D138N mutant were collected on beamline BL44B2 at SPring-8.
Structural and Functional Analyses of Drp35 from S. aureus (Harima, Japan) and on beamline NW12 at Photon Factory (Tsukuba, Japan), respectively. All of the diffraction data were indexed, integrated, scaled, and merged using the HKL2000 program package (16). The data collection and processing statistics are summarized in Table 1.

Structure Solution and Refinement—The initial phasing was achieved by the SAD method with the program SHELX (17). The subsequent phase improvement was performed with the programs SOLVE/RESOLVE (18–22) and DM (23–25). SHELX identified the positions of all 60 selenium sites. The program SOLVE/RESOLVE was used for heavy atom refinement and initial phasing. The initial electron density map was obtained after phase improvement by the program DM with the operator of noncrystallographic symmetry obtained by RESOLVE. A total of 2756 of 3996 residues were built automatically by RESOLVE, and 3792 residues were rebuilt manually using the program LSQKAB (26) as part of the CCP4 suite (27) and the graphics program O (28), based on the initial electron density map. The additional model building, positional energy minimization, and individual B factor refinements were carried out automatically with the program LAFIRE (29). To monitor the refinement, a random 10% subset from all reflections was set aside for calculation of the free R factor (R_free). After automatic refinement and model fitting by LAFIRE, several cycles of refinement with the program CNS (30) and manual model fitting were carried out, and then the water molecules were located automatically. Due to the abundance of residues in an asymmetric unit against observed reflections, the difference between R and R_free factor remarkably increased without the noncrystallographic symmetry restriction even in the final step of refinement. Therefore noncrystallographic symmetry restriction was applied to only the regions in which atoms could be well imposed throughout the refinement. The average of r.m.s. deviation of 12 molecules is 0.144 Å. Finally, 3849 residues and 1295 water molecules could be placed in the structure of Se-Drp35 with crystallographic R values and R_free values of 20.0 and 22.2%, respectively.

The structure of Drp35-Ca\(^{2+}\) complex and D138N mutant were determined by the molecular replacement method using the program MOLREP (31) using the structure of Se-Drp35 as a search probe. To monitor the refinement, a random 10% subset from all reflections was set aside for calculation of the R_free factor. The positional and individual B factor refinements were carried out automatically with the program LAIFIRE. After automatic refinement and model fitting by LAIFIRE, several cycles of refinement with the program CNS and manual model fitting were carried out. Finally, the water molecules were picked automatically by the program LAIFIRE, and then calcium ions and ligand molecules were placed manually. The crystallographic R values and R_free values for the Drp35-Ca\(^{2+}\) complex and Drp35-D138N mutant converged to 16.9% (19.7%) and 16.8% (20.2%), respectively.

The stereochemical qualities of the final refined models were analyzed using the program PROCHECK (32). The refinement statistics are summarized in Table 1. Although crystals of Drp35-Ca\(^{2+}\) complex and Drp35-D138N mutant belong to space group P2\(_1\) in analogy with Se-Drp35, none of the relationship was observed in cell parameters. The packing of noncrystallographically related molecules also does not have a simple correlation.

Enzyme Assay—Lactonase activity was determined as described previously using dihydrocoumarin as the substrate (6). The kinetic parameters for Drp35 were determined in the range of 4.1–9.0 from Lineweaver–Burk plots. Buffers used were acetate (pH 4.1–5.5), MES (pH 5.6–6.8), and Tris-HCl (pH 7.4–9.0). k\(_{cat}\)/K\(_{m}\) values for each pH value ((k\(_{cat}\)/K\(_{m}\))\(^H\)) were fitted to a bell-shaped model using the equation, (k\(_{cat}\)/K\(_{m}\))\(^H\) = (k\(_{cat}\)/K\(_{m}\))\(^{max}\)((10^{-pH}/10^{-pK_a1}) + (10^{-pK_a2}/10^{-pH}) + 1), where (k\(_{cat}\)/K\(_{m}\))\(^{max}\) is the pH-independent value, and pK\(_{a1}\) and pK\(_{a2}\) are the apparent pK\(_a\) values for the acidic and basic groups, respectively.

Differential Scanning Calorimetry (DSC)—All DSC measurements were carried out with a VP-CAPILLARY DSC SYSTEM (MicroCal, Northampton, MA). Proteins were dialyzed against 50 mM acetate, pH 5.6, and 1 mM EDTA or against 50 mM acetate, pH 5.6, and 1 mM CaCl\(_2\). The dialysis buffer was used as a reference solution for the DSC scan. Protein samples of 0.60–0.94 mg ml\(^{-1}\) were heated from 10 to 85 °C at a scanning rate of 1 K min\(^{-1}\).

RESULTS

Crystal Structure of Se-Drp35 and Drp35-Ca\(^{2+}\) Complex—The crystal structure of selenomethionine-substituted Drp35 was determined at a resolution of 2.4 Å by the SAD method (Table 1). This structural information enabled a molecular replacement method to determine the structure of the Drp35-Ca\(^{2+}\) complex at a resolution of 1.72 Å. The subunit structures of the Drp35-Ca\(^{2+}\) complex are well superposed with those of Se-Drp35 with an averaged r.m.s. deviation of 0.30 Å.

Each monomer consists of five helices (α1–α5) and 25 β-strands (β1–β25). These β-strands form six β-sheets, five of which are composed of four β-strands and one of which is composed of five β-strands (Fig. 1A). These β-sheets are located in a circular arrangement, resulting in a six-bladed β-propeller structure. As is often the case with many kinds of β-propeller protein, a “molecular clasp” tethering the N- and C-terminal regions within a β-sheet was also observed in two β-sheets (blades 5 and 6) in Drp35 (33). Interestingly, in all molecules of Se-Drp35, Drp35-Ca\(^{2+}\) complex, and Drp35-D138N mutant (see below), cis-peptide conformation was observed in His\(^{232}\)-Glu\(^{233}\), located in the connecting loop between blades 1 and 6 (Fig. 1A).

A structural alignment of Drp35 against all proteins in the Protein Data Bank by secondary structure matching (SSM) showed that the structure of Drp35 was similar to that of diisopropylfluorophosphatase (DFPase) from Loligo vulgaris and serum paraoxonase 1 (PON1) (r.m.s. deviation of 1.87 Å for 264 C\(_\alpha\) atoms and 2.50 Å for 237 C\(_\alpha\) atoms, respectively), although there was not significant amino acid sequence similarity (BLAST E-scores 4.3 and 1.6 for DFPase and PON1, respectively) (13, 34). These two eukaryotic proteins are classified as phosphotriesterases (EC 3.1.8) and possess a common biological activity (i.e. calcium-dependent hydrolysis activity) (9, 35). Similarly, Drp35 can hydrolyze lactones in a calcium-dependent manner and is a functional counterpart of PON (i.e. Drp35 is related structurally and functionally to DFPase and PON).
The cis-peptide conformation found in Drp35 is not observed in the corresponding region in DFPase and PON1. Another apparent structural difference of Drp35 from PON is the absence of a canopy composed of α-helices that is required for binding of PON to the lipid layer of high density lipoprotein (13). This is consistent with the observation that Drp35 is a soluble cytosolic protein and is not detected in the membrane fraction (6).

The reaction mechanisms for DFPase and PON have been reported. In both enzymes, a pocket at the center of the β-propeller acts as a substrate entrance tunnel in which substrates are captured and reacted. Drp35 also has a pocket at the identical position (Fig. 1C), suggesting that it would hold the active site (see below).

In the structure of Drp35-Ca\(^{2+}\) complex, two calcium ions (Ca1 and Ca2) were bound to one molecule of Drp35 (Fig. 1A). The averaged distance between Ca1 and Ca2 is 17.0 Å. Ca1 was bound at the bottom of the pocket located at the center of the β-propeller, where eight oxygen atoms derived from the side chains of Glu\(^{48}\), Asp\(^{138}\), Asn\(^{185}\), Asp\(^{236}\), Ser\(^{237}\), and three water molecules coordinated to Ca1 (Fig. 1B and C). One of the water molecules was bound not only to Ca1 but also to Oδ in Asp\(^{138}\). The average distances between Ca1 and each ligated oxygen atom were as follows: 2.48 Å for Oε in Glu\(^{48}\), 2.52 Å for Oδ in Asp\(^{138}\), 2.37 Å for Oδ in Asn\(^{185}\), 2.36 Å for Oδ in Asp\(^{236}\), 2.41 Å for Oγ in Ser\(^{237}\), and 2.56, 2.42, and 2.38 Å for three water molecules. The other calcium, Ca2, is bound on the surface, in which the carbonyl oxygens from Thr\(^{133}\), Ser\(^{110}\), Asp\(^{130}\), Tyr\(^{135}\), and Gly\(^{112}\) and Oγ from Thr\(^{133}\) were bound to Ca2. The average distances between Ca2 and each ligated oxygen atom were as follows: 2.55 Å for oxygen inThr\(^{133}\), 2.30 Å for oxygen in Ser\(^{110}\), 2.52 Å for oxygen in the main chain of Asp\(^{130}\), 2.50 Å for oxygen in Tyr\(^{135}\), 2.47 Å for oxygen in Gly\(^{112}\), and 2.51 Å for Oγ in Thr\(^{133}\). Although all coordinating atoms were oxygens as in Ca1, none of the carboxylate groups, which are the most frequently observed groups as ligands in Ca\(^{2+}\) protein complexes (36), participated in the Ca2 binding.

In crystal structures of DFPase and PON1 reported previously, a catalytically important calcium ion was observed at the identical positions to Ca1. The structural comparison of the Ca1 binding site in Drp35 with those in DFPase and PON1 shows that they have similar coordination geometries (Fig. 2), where three of five residues bound to Ca1 (i.e. Glu\(^{48}\), Asn\(^{185}\), and Asp\(^{236}\)) were conserved among the three proteins, Drp35, DFPase, and PON1 and Glu\(^{21}\), Asn\(^{175}\), and Asp\(^{229}\) in DFPase, respectively. However, two other residues, Asp\(^{138}\) and Ser\(^{237}\), were not conserved. Ser\(^{237}\) in Drp35 is replaced by Asn\(^{270}\) in PON1, and none of the residues coordinated here in DFPase. Asp\(^{138}\) in Drp35 correspond to Glu\(^{53}\), Asn\(^{224}\), and Asp\(^{269}\) in PON1 and Glu\(^{21}\), Asn\(^{175}\), and Asp\(^{229}\) in DFPase, respectively. However, two other residues, Asp\(^{138}\) and Ser\(^{237}\), were not conserved. Ser\(^{237}\) in Drp35 is replaced by Asn\(^{270}\) in PON1, and none of the residues coordinated here in DFPase. Asp\(^{138}\) in Drp35 is replaced with Asn in both PON1 and DFPase. DFPase and PON1 possess a structural calcium ion in addition to the catalytic calcium at an identical position, near the center of the β-propeller (13, 34). The distances between these two calcium ions are 7.4 and 9.4 Å, respectively. However, no calcium ion was observed at the corresponding site in Drp35.

Structural and mutational analyses suggested that His\(^{287}\) and Glu\(^{37}\) around the calcium binding site are catalytic residues in

### Table 1

| Data collection                                      | Selenomethionine-substituted structure (SAD) | Calcium complex | D138N mutant |
|------------------------------------------------------|---------------------------------------------|-----------------|--------------|
| Space group                                          | P2\(_1\)                                    | P2\(_1\)        | P2\(_1\)     |
| α = 86.09, b = 146.06, c = 151.95, β = 93.39         | a = 76.53, b = 181.98, c = 81.80, β = 115.41 | a = 76.55, b = 182.43, c = 81.45, β = 115.58 |
| Beamline                                             | Photon Factory NW12                         | Photon Factory NW12 |
| Resolution (Å)                                       | 50-2.40 (2.49-2.40)                         | 50-1.72 (1.78-1.72) |
| Wavelength (Å)                                       | 0.97908                                     | 0.90000         |
| Rsym (%), Rfree (%)                                   | 10.3 (46.6)                                 | 6.3 (39.5)      |
| Complement (%)                                       | 100 (100)                                   | 99.2 (95.0)     |
| Observations                                        | 1,561,878                                   | 806,546         |
| Unique reflections                                   | 145,982                                     | 211,193         |
| Multiplicity*                                        | 10.3 (10.2)                                 | 3.8 (3.4)       |

**Refinement and model quality**

| Resolution range (Å)                                 | 20-2.4                                      | 20-1.72         |
| No. of reflections                                   | 138,199                                     | 15,076          |
| R factor*                                            | 0.200                                       | 0.169           |
| Rfiter*                                              | 0.222                                       | 0.197           |
| Reffiter (%)                                         | 0.005                                       | 0.005           |
| Total protein atoms                                  | 30,096                                      | 15,082          |
| Total ligand atoms                                   | 0                                           | 84              |
| Total water atoms                                    | 1295                                       | 2141            |
| Solvent content (%)                                  | 42.0                                        | 46.7            |
| Average B factor (Å\(^2\))                           | 39.4                                        | 18.8            |
| r.m.s. deviation from ideality                        | 26.3                                        | 3.0             |

**Ramachandran plot**

| Residues in generously allowed regions (%)           | 0.8                                         | 0.4             |
| Residues in most favored regions (%)                 | 81.2                                        | 84.2            |
| Residues in additional allowed regions (%)           | 18.0                                        | 15.3            |
| Residues in generously allowed regions (%)           | 0.8                                         | 14.4            |

* The values in parentheses refer to data in the highest resolution shell.

* Rsym = \(\frac{\sum|I_o| - \langle I_o \rangle}{\sum|I_o|}\), where \(\langle I_o \rangle\) is the mean intensity of a set of equivalent reflections.

* R factor = \(\frac{\sum|F_o| - |F_c|}{\sum|F_o|}\) where \(F_o\) and \(F_c\) represent observed and calculated structure factor amplitudes, respectively.

* Reffiter = calculated for R factor, with a random 10% subset from all reflections.
DFPase (34, 37). In PON1, two histidine residues, His115 and His134, have been suggested to be catalytic residues, acting as a general base and proton donor, respectively (13, 15). Fig. 2 shows the catalytic residues in DFPase (Fig. 2B) and PON1 (Fig. 2C) and residues at corresponding positions in Drp35 (Fig. 2A). Interestingly, none of the catalytic residues in DFPase and PON1 are conserved in Drp35; His^{287} and Glu^{37} in DFPase are replaced with Asn^{299} and Phe^{64}, and His^{115} and His^{287} in PON1 are replaced with Ala^{90} and Leu^{105}. These results strongly suggest that Drp35 has a catalytic mechanism distinct from two other functionally and structurally related proteins.

**Effects of Mutations into Conserved Residues on Lactonase Activity**—To acquire insight into the active site of Drp35, we analyzed the structures of crystals grown or soaked in buffer containing the competitive inhibitor compounds, coumarin, benzamidine, and 8-quinolinol. However, we could not detect the clear electron density derived from the inhibitor compounds in any case despite the obvious electron density of Ca^{2+}. Then we focused on residues conserved among proteins homologous to Drp35 in other bacteria. A sequence alignment among seven homologous proteins proposed 27 residues as completely conserved residues (Fig. 3). Alanine-substituted mutant proteins of these residues were prepared, and their lactonase activities were compared. There were problems in preparation of three mutants, Gly^{231}, Asp^{241}, and Val^{248}. In the case of Gly^{252}, the G252V mutant was used, because we could not construct an expression vector for G252A for unknown reasons. Fig. 4A shows the activity of each mutant protein relative to wild-type Drp35. Some fluctuations were found in the activities of several mutant proteins, as is also the case with PON (14). Marked decreases were observed in six mutants: E48A, D139A, D152A, N185A, G186A, and D236A. As described above, Glu^{48}, Asn^{185}, and Asp^{236} directly coordinated to Ca1 in the structure of the Drp35-Ca^{2+} complex. In addition, Asp^{139} and Gly^{186} are neighboring residues to Ca^{2+}-binding residues, Asp^{138} and Asp^{185}. The distances between C9 atoms of Asp^{139} and Gly^{186} and Ca1 are 5.94 and 5.28 Å, respectively. Although Asp^{152} is neither a Ca1-binding residue nor a neighboring residue, it is located rather close to Ca1; the distance between the C9 atom of Asp^{152} and Ca1 is 10.7 Å. These results suggest the importance of Ca1 for catalytic activity of Drp35, as reported for calcium ion binding at the identical position in DFPase and PON1. This was strongly supported by the pocket structure of Drp35, which is generally observed for substrate binding sites in enzymes (38). Ca1 binding at the bottom of the pocket also suggests a connection between the possible active site and the substrate entrance tunnel (Fig. 1C).

**Effects of Mutations into Residues around the Calcium Binding Site**—We investigated residues located around the Ca^{2+}-binding site by alanine scanning (Fig. 4B). Mutated residues could be classified into three groups: residues participating directly in binding to Ca1 (gray bars in Fig. 4B), those comprising the tunnel described above (closed bars in Fig. 4B), and those located in the interior of the protein, where structural

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**FIGURE 1. Crystal structure of Drp35-Ca^{2+} complex.** A, ribbon diagram of the Drp35-Ca^{2+} complex. The ribbon model is colored according to the sequence from blue at the N terminus to red at the C terminus. Two calcium ions binding to Drp35 (Ca1 and Ca2) are shown as pink balls. The cis-conformation residues, His^{322}, Glu^{313}, are shown as sticks. B, close-up view of the Ca1 binding site. Ca1 is shown as a pink ball, and residues coordinating to Ca1 are shown as sticks. Water molecules binding to Ca1 are also shown as blue balls. C, a surface view of the pocket in which Ca1 is bound at the bottom. Red and blue represent negative and positive charges, respectively. Two calcium ions are shown as green balls. Phe^{64} is also shown as orange balls.
calcium was bound in DFPase and PON1 (open bars in Fig. 4B). A comparison of lactonase activity showed that significant inactivation occurred by replacing residues that bind directly to Ca1. The extent of the decrease in the Ser\textsuperscript{237} to Ala mutant was weaker, although for the other four Ala mutants the decrease was striking. Mutations in residues categorized into two other groups did not bring about noticeable decreases in activity except for Phe\textsuperscript{153}, in which the activity was 7.6% of wild type. It should be noted that lactonase activity increased up to 2.5-fold that of the wild-type enzyme when Phe\textsuperscript{64}, which is the structural counterpart of the catalytic residue Glu\textsuperscript{47} at the entrance of the tunnel of DFPase, was substituted by alanine. In addition to F64A, increases in lactonase activity were observed in R281A, E302A, K93A, and C238A.

Effects of Substituting a Variety of Amino Acid Residues for Calcium-binding Residues—The results described above suggested that residues participating in calcium binding except Ser\textsuperscript{237} strongly influence the lactonase activity. To test whether the integrity of the coordinated network with the calcium ion is critical for lactonase activity, these residues were substituted to other residues as follows: Glu was substituted to Ala, Ser, and Gln; Asp was substituted to Ala, Ser, and Asn; and Asn was substituted to Ala, Ser, and Asp. Fig. 4C shows the resultant activities of these mutants relative to the wild-type enzyme. The replacement of Glu\textsuperscript{48}, Asp\textsuperscript{138}, and Asp\textsuperscript{236} with other amino acids led to complete abolition of the activity. In the case of Asn\textsuperscript{185}, which is a completely conserved residue in homologous proteins, mutations to Ala or Ser resulted in inactivation, but N185D maintained a certain degree of activity as the S237A mutant. Based on these results, the Ca1-binding residues could be divided into two groups according to their influence on the enzymatic activity; one group includes residues with a strong influence, Glu\textsuperscript{48}, Asp\textsuperscript{138}, and Asp\textsuperscript{236}, whereas the other includes Asn\textsuperscript{185} and Ser\textsuperscript{237}, the efficacy of which was not so strong.

Evaluation of the Calcium Binding of Drp35, E48Q, D138N, and D236N Mutants by Differential Scanning Calorimetry—To evaluate whether the substitutions in Glu\textsuperscript{48}, Asp\textsuperscript{138}, and Asp\textsuperscript{236} abolish binding to the calcium ion, the thermostabilities of wild-type Drp35, E48Q, D138N, and D236N were measured by differential scanning calorimetric assay (Fig. 5). Under control conditions without calcium ions, each protein exhibited similar thermograms with a peak between 55.8 and 58.2 °C (Fig. 5A). However, clear differences were observed in the presence of 1 mM CaCl\textsubscript{2} (Fig. 5B). The thermogram of the wild type exhibited a peak with the highest value of 70.4 °C, followed by D138N with a peak of 66.6 °C, D236N with a peak of 60.8 °C, and E48Q with a peak of 58.3 °C. The increases in $T_m$ value upon the addition of calcium ions were 12.2 °C for wild type, 9.6 °C for D138N, 5.0 °C for D236N, and 0.3 °C for E48Q. Since the increase in $T_m$ is thought to be due to the structural stabilization caused by calcium ion binding, these results suggest that 1) calcium ions and their coordinating residues are represented as green balls and sticks, respectively. The residues whose carbon atoms are colored green are conserved among three proteins. The catalytic residues in DFPase (B) and PON1 (C) and the residues located in identical positions to the catalytic residues of DFPase and PON1 in Drp35 (A) are shown as pink sticks. Asp\textsuperscript{152} and Phe\textsuperscript{153} are also shown.

**FIGURE 2.** Comparison of the Ca1 binding site in DFPase and PON1. A, Drp35 from S. aureus. B, DFPase from L. vulgaris (Protein Data Bank code 1E1A). C, serum paraoxonase 1 (Protein Data Bank code 1V04). The catalytic calcium ions were bound in DFPase and PON1 (open bars in Fig. 4B). A comparison of lactonase activity showed that significant inactivation occurred by replacing residues that bind directly to Ca1. The extent of the decrease in the Ser\textsuperscript{237} to Ala mutant was weaker, although for the other four Ala mutants the decrease was striking. Mutations in residues categorized into two other groups did not bring about noticeable decreases in activity except for Phe\textsuperscript{153}, in which the activity was 7.6% of wild type. It should be noted that lactonase activity increased up to 2.5-fold that of the wild-type enzyme when Phe\textsuperscript{64}, which is the structural counterpart of the catalytic residue Glu\textsuperscript{47} at the entrance of the tunnel of DFPase, was substituted by alanine. In addition to F64A, increases in lactonase activity were observed in R281A, E302A, K93A, and C238A.

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the marked decreases in enzymatic activity seen in E48Q and D236N are due to loss of the calcium ion and that 2) D138N can bind the calcium ion similarly to the wild type despite the loss of activity. Taken together with the observation that Asp138 is the calcium-binding residue that is not structurally conserved among Drp35, PON, and DFPase (Fig. 2), we considered that Asp138 may be involved in the characteristic catalytic mechanism of Drp35.

**Structural Analysis of D138N Mutant**—We solved the crystal structure of the D138N mutant. The crystallographic parameters are not changed from those of the Drp35-Ca2+ complex, and the asymmetric unit contained six molecules of D138N mutant protein (Table 1). Crystals were grown in buffer containing 5 mM calcium ions, and the catalytic calcium was clearly observed as in the wild type with sufficient occupancy in all six molecules, consistent with the results of differential scanning calorimetric analysis. There were no noticeable conformational changes for residues binding to the calcium ion except for the mutated residue, Asn138 (Fig. 6). The carbonyl group in the side chain of Asn138 was rotated by about 15° compared with Asp138 in the wild-type structure. It is obvious that the occupancy of water1 with the other two water molecules or that observed in the asymmetric unit, although two other water molecules that were assigned to any compound at the bottom of the tunnel. Inter- 

**pH Dependence of Enzyme Catalysis**—Acidic pk_a values of 5.4 and 6.7, corresponding to pK_a1 and pK_a2, in the equation, respectively. The acidic pk_a of 5.4 is, as expected, 2.0 and 1.4 lower than those reported previously for PON1 and DFPase, respectively. In the high pH environment, Drp35 exhibited a decrease in specific activity with an acidic pk_a value of 6.7 for as yet undetermined reasons, which was also observed for PON1, where the pk_a value was 9.8.

**DISCUSSION**

**Active Site and Deduced Catalytic Residue**—The three-dimensional structure of Drp35, a six-bladed β-propeller structure, is similar to those of DFPase and PON1, which are functionally related proteins despite the lack of significant amino acid sequence similarity (13, 34). These proteins have the common enzymatic feature of calcium dependence (9, 35). Two calcium ions were observed in the Drp35-Ca2+ complex, one of which (Ca1 in Fig. 1) was present in the identical position in two other proteins (i.e. the center of the β-propeller). From the results of the present study, we concluded that the Ca1 binding site constitutes an active site, as is the case in both DFPase and PON1.

As described above, we could not reveal any structure complexed with inhibitors. However, in these structures, the F_o − F_c map showed a certain electron density that could not be assigned to any compound at the bottom of the tunnel. Interestingly, this electron density was surrounded by Asp138, Asp236, and the calcium ion (data not shown). In analogy with the other enzymes whose active site can be easily complexed with certain compounds (13, 39 – 41), this observation also supports our conclusion that the active site is positioned around the calcium ion.

The structural comparison focusing on the residues binding to the catalytic calcium among Drp35, DFPase, and PON1 revealed that coordination of the calcium ion was also quite
similar, and three of five residues binding to the catalytic calcium ion, Glu48, Asn185, and Asp236, are conserved among the three proteins (Fig. 2). Despite these structural similarities, the catalytic residues identified in DFPase and PON1 do not exist in Drp35 (Fig. 2), suggesting that the catalytic mechanism of Drp35 is distinct from these enzymes. This was strongly supported by the enhancement, rather than reduction, of the enzymatic activity due to the substitution of Phe64, which is the structural counterpart of the catalytic residue in DFPase (Figs. 2 and 4B).

The five residues coordinating to the catalytic calcium could be divided into two groups; Glu48, Asn185, and Asp236 are structurally conserved in PON and DFPase, whereas Asp138 and Ser237 are not. On the other hand, mutations in Glu48, Asp138, and Asp236 resulted in marked inactivation irrespective of the substitution residues, although the influences of mutations in Ser237 and Asn185 were rather weak. DSC analysis revealed that the marked inactivation in E48Q and D236N was due to loss of the calcium ion. It was also reported for DFPase that E21Q and D229N, which correspond structurally to Glu48 and Asp236 in Drp35, lacked the calcium ion (34). In contrast, the results of DSC and structural analysis of D138N indicated that the decrease in catalytic activity was not due to the loss of the calcium ion and was probably due to the disappearance of one water molecule, designated water1. These results suggest a probable catalytic mechanism involving water1 (see below), and its binding would be finely tuned by Asp138.

Thus, Asp138 is an intriguing residue in that it is not conserved structurally among the three proteins but is strongly correlated with the catalytic activity of Drp35. Considering the absence in Drp35 of the catalytic residues in PON1 and DFPase, Asp138 seems to specify the unique catalytic mechanism of Drp35. This prediction may also be supported by the acidic pKα value of 5.4. Asp139 and Asp152, of which substitution for Ala caused significant inactivation (Fig. 4A), can be other candidates for this pKα value. Although these locate around Ca1, they would not be the catalytic residue, because 1) Asp139 is located behind Ca1, and it

FIGURE 4. Changes in lactonase activity by mutation. The activity of each mutant protein relative to the wild-type enzyme is shown. A, results of mutation in completely conserved residues. B, results of mutation in residues located around Ca1. Enzymes with mutations of the residue binding directly to Ca1, those composing the tunnel leading toward Ca1, and those located in the interior of the protein are shown as gray, closed, and open bars, respectively. C, the results of mutations in the residues coordinating to Ca1.

would not be able to interact with a substrate directly unless catalytic $\text{Ca}^{2+}$ goes away, and 2) the side chain of Asp$^{152}$ does not face the bottom of the pocket in which other essential residues and Ca1 are located. The mechanism responsible for the basic $pK_a$ value of 6.7 is unclear, but it indicates that the solved structure should represent the form inactivated by the basic environment, because crystallization was performed at pH higher than 6.7. The coordination of hydroxyl groups of the water molecules to the catalytic calcium may be affected by the pH value to influence the activity. It should also be noted that the $pK_a$ value of 6.7 would not be attributable to imidazole groups, because mutations in any of the histidine residues located around the active site did not cause significant inactivation (Fig. 4).

Proposed Reaction Mechanism of Drp35—Based on the findings described above, we propose a possible reaction mechanism of Drp35 as follows (Fig. 8). First, the water molecule binding to Ca1 and Asp$^{138}$ is activated by deprotonation. The substrate enters the tunnel and binds to Ca1, and the activated water molecule is then located beside the substrate. The water would attack the carbon atom in the carbonyl group of the substrate and cause hydrolysis. Protonation accompanied by hydrolysis is thought to be mediated by Asp$^{236}$, as suggested by the steric arrangement around Ca1. This was also supported by the observation that the unassigned electron density beside the Ca1 is surrounded by Asp$^{138}$, Ca1, and Asp$^{236}$. Although the $pK_a$ value of the carboxyl group in proteins is usually within the range from 2 to 5.5, it was reported that the aspartic acid residue acts as a proton donor with a $pK_a$ value of 6.5 and 7.8 in two calcium-dependent hydrolases, nucleoside hydrolase and $\alpha$-mannosidase, respectively (42, 43). In analogy with this, it is possible to suppose that Asp$^{236}$ is protonated within the pH range optimal for the catalytic activity of Drp35, embedded in its unusual environment.
The calcium ion is found in proteins, such as calmodulins and other Ca$^{2+}$ sensor proteins (44), and it plays a role in structure-forming switching control. It is also involved in stabilizing protein structure (35, 45, 46). In addition, calcium ion plays roles in substrate binding or catalytic reaction in some enzymes, including DFPase (34), PON1 (15), glycosidase (43), nuclease (42, 47), and phospholipase A2 (48). It should be noted that the proposed catalytic mechanism for Drp35 is similar to the established mechanism in nucleoside hydrolase (49), \(\alpha\)-mannosidase (43), and glucoamylase, where a water or carboxyl group coordinating to the calcium ion indeed participates directly in the hydrolytic reaction. Nucleoside hydrolase has been studied extensively by structural and biochemical analyses, and it was reported that the water molecule bound to both the catalytic calcium and \(O_6\) from Asp10, which also bound to catalytic calcium via the other \(O_6\) atom, is activated and attacks the substrate (49). Protonation of intermediates by the carboxyl group binding the catalytic calcium via \(O_6\) was recently identified for glucoamylase.\(^3\) In this enzyme, two carboxyl groups binding directly to calcium act as catalytic residues, and the calcium ion directly binds the substrate. One carboxyl group, which acts as a general base, activates the water molecule essential for nucleophilic attack, and the other one, which acts as a general acid, protonates the intermediate. These reports also constitute a rationale for the Asp138-dependent mechanism proposed for Drp35. Although such a reaction mechanism has been reported previously for only a few proteins, it may be a typical mechanism of hydrolysis.

In the proposed model, the calcium ion plays the critical role in the catalysis. This is consistent with the fact that EDTA inhibits the activity of Drp35 (6). It is known that some enzymes, such as nucleoside hydrolase and \(\alpha\)-mannosidase, specifically require the Ca$^{2+}$ (50, 51), whereas it can be substituted with other cations without abolishing the catalytic activity in phospholipase A2 (48). Drp35 is also compatible with other divalent cations, such as Mg$^{2+}$ or Mn$^{2+}$ (data not shown). Elucidation of the catalytic mechanism(s) involving such distinct kinds of cations will be important future work to know the cation specificity of this catalysis model.

**Biological Implications**—Drp35 is thought to be related to stress adaptation or clearance, and it apparently possesses lactonase activity (6). However, it is still unclear how this activity contributes to the mechanisms of coping with stresses, and the nature of the genuine substrate in vivo has yet to be determined. The F64A mutant of Drp35 showed 2.5-fold higher activity than the wild-type enzyme. Phe$^{64}$ is located at the entrance of the tunnel that connects to the active site. This would reflect the disagreement of the substrate used for measuring enzymatic activity (i.e., dihydrocoumarin) with its true substrate in vivo. The activation may be caused by fortuitous optimization of the structure for dihydrocoumarin. Replacement of the bulky phenylalanine residue with alanine may cause increases in plasticity and the dimensions of the entrance. The true substrate may be a smaller or stalker compound than 3,4-dihydrocoumarin that could easily reach the catalytic center even in the wild-type enzyme. The negatively charged surface of the tunnel also suggests that the substrate would have a positive charge. In the future, extensive compound screening should be conducted to identify the in vivo substrate. This will provide critical insight into the function of Drp35 and facilitate the development of means to overcome hospital-acquired S. aureus infection.

**Conclusions**—Structural analyses of Drp35 revealed a six-bladed \(\beta\)-propeller structure with a calcium ion at the center. Mutation analyses indicated the significance of the calcium ion and its coordinating residues. The crystal structure of the DI38N mutant showed the disappearance of a water molecule in the active site. Based on these findings, a possible mechanism was proposed for the lactonase activity in which the calcium ion and the coordinating aspartate residues participate.

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**FIGURE 8. Schematic representation of proposed mechanism for lactonase activity of Drp35.** A water molecule bound to Ca1 and Asp138 is activated by Asp138 and Ca1 (left). The generated hydroxyl group attacks the carbon atom in the carbonyl group of the substrate, and the oxygen atom whose covalent bond is broken is protonated by Asp236 (center). 3-2-Hydroxynaphthalpropionic acid is generated.
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