Crystal Structure of Phosphorylcholine Esterase Domain of the Virulence Factor Choline-binding Protein E from Streptococcus pneumoniae

NEW STRUCTURAL FEATURES AMONG THE METALLO-β-LACTAMASE SUPERFAMILY*

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Streptococcus pneumoniae is the worldwide leading cause of deaths from invasive infections such as pneumonia, sepsis, and meningitis in children and the elderly. Nasopharyngeal colonization, which plays a key role in the development of pneumococcal disease, is highly dependent on a family of surface-exposed proteins, the choline-binding proteins (CBPs). Here we report the crystal structure of phosphorylcholine esterase (Pce), the catalytic domain of choline-binding protein E (CBPE), which has been shown to be crucial for host/pathogen interaction processes. The unexpected features of the Pce active site reveal that this enzyme is unique among the large family of hydrolases harboring the metallo-β-lactamase fold. The orientation and calcium stabilization features of an elongated loop, which lies on top of the active site, suggest that the cleft may be rearranged. Furthermore, the structure of Pce complexed with phosphorylcholine, together with the characterization of the enzymatic role played by two iron ions located in the active site allow us to propose a reaction mechanism reminiscent of that of purple acid phosphatase. This mechanism is supported by site-directed mutagenesis experiments. Finally, the interactions of the choline binding domain and the Pce region of CBPE with chains of teichoic acids have been modeled. The ensemble of our biochemical and structural results provide an initial understanding of the function of CBPE.

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** The abbreviations used are: PCho, phosphorylcholine; CBP, choline-binding protein; CBD, choline binding domain; Pce, phosphorylcholine esterase; 5NP-PC, p-nitrophenyl-phosphorylcholine; MPD, 2-methyl-2,4-pentanediol; MME, monomethylether; DTT, dithiothreitol; PEG, polyethylene glycol; WT, wild type; Pap, purple acid phosphatase.

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(CBPs) by the means of choline binding domains (CBDs) (21, 22). Up to 15 CBPs have been identified in pneumococcal strains (CBPA–G, LytA–C), each containing a CBD located at the N or C terminus, characterized by the repetition of a 20-residue consensus motif GWXXWYYXXGXX(4, 5, 23). In addition, CBPs harbor a domain of variable length to which enzymatic properties have been associated in some instances: LytA, LytB, and LytC cleave peptidoglycan bonds, a function also proposed for CBPDE, whereas CBPG has been proposed to be a serine protease (24–28). A role in pneumococcal virulence has been attributed for almost all CBPs: PcpA is thought to be involved in protein-protein and protein-lipid interactions, whereas PsPA inhibits complement activation, among other effects (Refs. 7 and 29 and references therein). CBPA is a cell surface adhesive and plays a major role in colonization of the nasopharynx by binding to immobilized sialic acid and lacto-N-neotetraose on cytokine-activated human cells (30). CBPA also interferes with the host immune response by interacting with the polymeric Ig receptor, the C3 complement component, and the complement-control protein factor H (Refs. 7, 12, and 31 and references therein). Finally, CBPD, CBPE, and CBPG have been shown to be involved in nasopharynx colonization.

Mutant strains deleted in the cbpE and cbpG genes present, in addition, a significantly lower ability to adhere to human epithelial cells (23). Increasing knowledge regarding nasopharynx colonization and tissue invasion processes has highlighted the participation of surface-associated proteins in both stages of pneumococcal disease, and such molecules are becoming of major interest as potential new antibiotics targets and/or vaccine candidates, as already established for the CBPs (32).

In this work, we focused on CBPE, one of the most important members of the CBP family. The CBD of CBPE is composed of 10 repeats, and the N-terminal region is predicted to harbor a domain with a metallo-β-lactamase fold (33). This domain, referred to as phosphorylcholine esterase (Pce), catalyzes the hydrolysis of the choline-phosphoester bond, releasing PCho molecules from cell wall-associated teichoic and lipoteichoic acids (34–36). A pneumococcal mutant deleted in cbpE is much less virulent than the wild-type strain in colonizing the nasopharynx, harbors a decreased ability to adhere to nasopharyngeal cells, but causes an increase in virulence in a model of intraperitoneal inoculation (23, 34). Here, we present the three-dimensional structure of Pce to a resolution of 2 Å. The features of the Pce active site show that this enzyme is unique among the large family of hydrolyases harboring the metallo-β-lactamase fold. Indeed, the orientation and calcium stabilization characteristics of an elongated loop, which lies on top of the active site, suggest that the cleft may be rearranged. Furthermore, the complexed structure of Pce with PCho, together with the characterization of catalytic iron ions in the active site, led us to propose a reaction mechanism that is supported by site-directed mutagenesis.

**MATERIALS AND METHODS**

**Cloning of the pce Gene Encoding the Catalytic Domain of CBPE**—Genomic DNA from the 6 strain of *S. pneumoniae* was used as a template to amplify the pce gene by conventional PCR methodology. Subsequently, the resulting product was cloned into the pHIS8 vector (pHIS8/pce). This construct encodes the Pce domain of CBPE (Glu2–Ser214), deleted from the peptide signal and from the CBD, fused to a Hisa tag at the N terminus. DNA sequencing (Genome Express, Grenoble) confirmed that no mutations had been introduced during PCR.

**Mutagenesis of the pce Gene**—The pHIS8/pce construct was employed as a template for QuikChange™ site-directed mutagenesis (Stratagene) following the manufacturer's instructions. The D62A, D114N, and H253N-encoding constructs were also subsequently sequenced (Genome Express), and no mutations other than the ones expected were introduced during PCR.

**Purification of Wild-type and Mutant Pce Proteins**—An overnight culture of *Escherichia coli* BL21(DE3) strain transformed with pHISS/ pce was used to inoculate (1:50) 1 liter of Luria Bertani medium supplemented with 50 μg/ml of kanamycin. On achieving an optical density at 600 nm equal to 1 at 37 °C, protein expression was induced with 1 mM isopropyl β-D-thiogalactopyranoside while incubating for 16 h at 15 °C. Cells were centrifuged at 6,000 × g for 5 min, the pellet was resuspended in 80 ml of a solution of 50 mM Tris-HCl, pH 8.0, 200 mM NaCl, 20 mM imidazole (buffer A), containing 2 tablets of Complete protease inhibitor mixture (Roche Applied Science). Cells were sonicated, the cell lysate was centrifuged at 31,000 × g for 20 min, and the resulting supernatant was loaded onto a 5-ml chelating Sepharose column (Amersham Biosciences), previously loaded with 100 mM NiSO4 and equilibrated in buffer A. Extensive wash steps (10 column volumes) with buffer A containing successively 50 and 100 mM imidazole were performed preceding the elution of the protein with 300 mM imidazole (buffer B); subsequently, the sample was extensively dialyzed in 50 mM Tris-HCl, pH 8.0, 50 mM NaCl. Pce was incubated with 4 units of thrombin per mg of fusion protein for 1 h at room temperature to cleave the N-terminal His tag. The cleaved product was loaded onto an anion-exchange chromatography column (Resource Q; Amersham Biosciences) at a flow rate of 2 ml/min, and a NaCl linear gradient (0–300 mM) was applied to elute Pce. Finally, gel filtration was performed on Superdex 200 HR 10/300 column. The three Pce samples in 20 mM ammonium acetate, pH 6.0; native forms were recombinant forms; were analyzed by SDS-PAGE electrophoresis and ESI-mass spectrometry.

**Native Mass Spectrometry Characterization of the Apo and Reconstituted Forms of Pce**—Noncovalent mass spectrometry measurements were performed by using Q-TOF Micro mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray ion source. It was operated with a needle voltage of 2.7 kV, sample cone and extraction cone voltages, respectively, of 150 V and 10 V, backing Pirani pressure was 600 mTorr. The mass spectra were recorded in the 2000–4000 mass-to-charge (m/z) range. Samples were continuously infused at a flow rate of 7 μl/min, data were acquired in the positive mode, and calibration was performed using a solution of 0.5 mg/ml CaI in water/isopropyl alcohol (1:1, v/v). Data were processed with MassLynx 4.0 (Micromass). The apo form of Pce deleted from the His tag was prepared as follows: 20 mM EDTA was added to the protein (20 μM) and incubated for 2 h at room temperature; the protein solution was subsequently dialyzed against 20 mM ammonium acetate, pH 6.0. Apoprotein was then incubated with 1 mM Fe2+, 5 mM dithiothreitol (DTT), 1 mM CaCl2, and 10 mM PCho for 2 h at room temperature. Protein solution was again extensively dialyzed against 20 mM ammonium acetate, pH 6.0 to eliminate unbound molecules, leading to the reconstituted Pce protein. The resulting samples in 20 mM ammonium acetate, pH 6.0, native (before treatment), apo, and reconstituted forms; were analyzed by mass spectrometry under non-denaturing conditions.

**pNP-PC Assays**—The PCho esterase activity of Pce wild type and mutants were measured with p-nitrophenyl-phosphorylcholine (pNP-PC, Sigma) as the substrate at 37 °C in 50 mM potassium phosphate buffer, pH 8.0, in a total volume of 200 μl. The activity was measured by following the increase in absorbance at 405 nm with the production of p-nitrophenol (pNP), whose quantification as a reaction product of Pce activity had been previously calibrated with a standard curve. Apo forms of wild-type and mutant proteins were prepared by incubation for 2 h at room temperature with 20 mM EDTA and subsequent extensive dialysis against 50 mM Tris, pH 8.0, 150 mM NaCl. The apo forms were used in the enzymatic assays with and without addition of iron, DTT, and calcium. Metal ions and DTT were added in concentrations from 0.01 to 1 mM. Reduced (Fe2+) and oxidized (Fe3+) forms of iron were generated from Fe(NH4)2(SO4)2 salt (uncolored solution in presence of 10 mM DTT) and FeCl3 (deep orange-colored solution), respectively. The concentration of pNP-PC varied from 0.16 to 32.8 mM, and each protein was used at concentrations around 70 μM. The kinetic parameters were determined by fitting the data to the Michaelis-Menten equation.

**Crystalization Data Collection and Processing**—A 40 mg/ml slightly brown solution of Pce in 25 mM Tris, pH 8.0, 100 mM NaCl was used to grow crystals at 15 °C using the hanging-drop method with a reservoir solution containing 48% (w/v) 2-methyl-2,4-pentanediol (MPD), 100 mM NaCl, 100 mM Tris, pH 8.5. Typically, the best crystals grew within a few days to dimensions of 200 × 200 × 50 μm. Similar crystals were obtained in
Crystal Structure of Phosphorylcholine Esterase

30% (w/v) PEG 550 monomethoxy ethyl (MME), 100 mM Tris, pH 8.5. A crystal obtained using MPD as precipitant was soaked for 1 day in 0.1 mM PCho, mounted in a loop, and flash-cooled in liquid nitrogen. X-ray data were obtained in-house using monochromatic CuKa x-rays supplied by a Nonius FR501 rotating-anode generator, coupled to a Mar Research Image Plate detector. Another crystal, obtained using PEG550 MME as precipitant, was first transferred to a cryoprotectant solution (reservoir solution containing 20% glycerol), mounted on a loop, and flash-cooled in liquid nitrogen. X-ray data at the Lα (remote) and Lβ (peak) edges of iron were collected at the ID29 ESRF beamline. Data sets were processed using MOSFLM and SCALA of the CCP4 program suite (37).

Structure Determination and Refinement—The structure of Pce was solved using the single wavelength anomalous dispersion method (38). Inspection of the in-house collected data set showed an anomalous signal, which could be interpreted as the anomalous signal of iron at 1.5416 Å (f’ = 2.2 e−). The positions of four iron atoms in the asymmetric unit were determined using SOLVE (39). Phase refinement resulted in an initial figure of merit of 0.24. Density modification with non-crystallographic symmetry averaging by RESOLVE (40) increased the figure of merit to 0.65 and gave a map of good quality. Automatic building using RESOLVE resulted in a model that included 76% of the sequence. Multiple rounds of model building with O (41) and refinement with REFMAC (42) generated the final model. Ca2+ assignments for the Pce structure were also obtained using the in-house data set, indicating a lower ligand occupancy. The refined structure obtained from this crystal, which was not soaked with Tyr83 and Val86, whereas the Phe65 and Pro66 side chains end of helix G3 (Fig. 2A). A network of hydrogen bonds between Tyr63–Asp89, Asp64–Trp152, and Glu60–His85 stabilizes the elongated loop (Fig. 2A). Tyr63 makes hydrophobic contacts with Tyr63 and Val186, whereas the Phe65 and Pro66 side chains form hydrophobic interactions with the side chains of Thr81 and Tyr152, respectively. The interface between the elongated loop and the rest of the protein is mainly hydrophilic and contains 8 buried water molecules. Taken together, the orientation of this elongated loop might modulate the shape of the PCho binding site and/or accessibility to the teichoic acid substrate.

Two Ca2+ ions and several electrostatic and hydrophobic interactions further stabilize the orientation of the elongated loop (Fig. 2A). The side chains of Glu41 and Asp62, together with the backbone oxygen of Asp62 and Asp64 and two water molecules, form the coordination sphere of the first Ca2+ ion (Fig. 2A). The second Ca2+ ion is located 6.3 Å away from the first: its coordination sphere is formed by the side chains of Asp62, Asp120, Glu121, two water molecules, and the backbone oxygen of Gly117 (Fig. 2A). Because of the importance of Asp62, which bridges the two Ca2+ ions, the mutant D62A was constructed, and the kinetic parameters were measured. As shown in Table II, no significant difference was observed between Pce and the mutant D62A, suggesting that the removal of one Ca2+ coordination site out of 6 does not induce major modifications. Despite the fact that the D62A mutant displays the same level of activity toward the synthetic substrate pNP-PC as the wild-type, Ca2+ ions are suggested to stabilize the elongated loop, whose position and orientation may play a role in the selectivity for the natural substrate of CBPE.

Active Site Structure—The active site of Pce is a 12-Å deep cavity mainly made up by iron ligands and by residues on the loops between β5-α3 (Arg146, Trp148), β8-β9 (Trp205, Asp206), and the Pce catalytic region (Glu27–Arg298) and the core of the protein (Glu27–Ser334) were found in the asymmetric unit (root mean square deviation of 0.13 Å for pairs of Cα atoms), 2 PCho molecules, a total of 359 water, and 7 MPD molecules were also detected (Table I). The Pce structure folds in a four-layer α/β sandwich structure, in accordance with it belonging to the metallo-β-lactamase superfamily fold (33, 43). The α-helices are exposed to the solvent and surround the compact core of the β-sheets (Fig. 1B). The catalytic region can be divided into two structural domains. The first half (Glu27–Asn171) is formed by three β-strands (β1β2β3) and three helix-strand elements (α1β4, α2β5, α3β6), whereas the second (Ala172–Arg298) is formed by four β-strands (β7β8β9β10), two helix-strand elements (ε4β11, α5β12), and one α-helix (α6). Finally, four short 310 helices (G1–G4) are clustered near the active site. An approximate internal molecular 2-fold symmetry is observed between the two halves, with the binuclear metal center located on the 2-fold axis. The linker region consists of β13, located in the second half of the catalytic region, and of β14, β15, and a short 310 helix (G5) in the first half (Fig. 1B).

The ϕ/ψ angles of the Asn51, Asp58, Trp148, Asp176, Glu200, and His254 residues are located in the disallowed areas of the Ramachandran plot. Asn51 (ϕ = 77°; ψ = −71°) belongs to the β2- and β3-strands. Asp58 (ϕ = 61°; ψ = 173°) interrupts the β3-strand and turns the protein backbone by about 90°, creating a hole filled by 3 water molecules inside the core of Pce. Trp148 (ϕ = 33°; ψ = −114°), lying in the active site, is a key residue in the interaction with PCHO, while Asp176 (ϕ = 1°; ψ = −48°) precedes the β3-strand that starts the second half of the catalytic region. Glu200 (ϕ = 23°; ψ = 111°) belongs to an external fragment (Glu192–Lys203) of the loop between β8 and β9. In both Mol A and Mol B, this fragment is characterized by a relatively high temperature factor and is located at the interface between the two molecules. Finally, His254 (ϕ = 72°; ψ = −62°) is one of the metal coordinating residues.

The Shape of the Pce Active Site Possibly Modulated by an Elongated Loop Stabilized by Ca2+ Ions—A striking feature of Pce, unique among reported structures of the metallo-β-lactamase superfamily, is the presence of an elongated loop, which lies on top of the active site (Fig. 2A). This loop, which joins the β3-strand to the α1-helix, starts at Glu60 and ends at Thr81. The terminal fragment, Gly78–Thr81, delimits the substrate binding pocket. Furthermore, Pro74 forces residues Arg76–Ile79 to approach Trp148, which, in a strained conformation, is at the end of helix G3 (Fig. 2A). A network of hydrogen bonds between Tyr63–Asp89, Asp64–Trp152, and Glu60–His85 stabilizes the elongated loop (Fig. 2A). Tyr63 makes hydrophobic contacts with Tyr63 and Val186, whereas the Phe65 and Pro66 side chains form hydrophobic interactions with the side chains of Thr81 and Tyr152, respectively. The interface between the elongated loop and the rest of the protein is mainly hydrophilic and contains 8 buried water molecules. Taken together, the orientation of this elongated loop might modulate the shape of the PCHO binding site and/or accessibility to the teichoic acid substrate.

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and β11-α5 (His253, His255, Asp256). The active site is also delineated by residues in the β1-β2 loop (Gly41, Gly42) and by residues in the elongated loop joining β3 to α1 (Gly78, Glu80, His85) (Fig. 2B).

**Iron Ions Are Essential for Catalysis**—The electron density map on Pce crystals indicated the presence of two metals in the active site. From in-house data collection, the anomalous diffraction signal observed with CuKα x-rays ruled out the presence of zinc but cannot discriminate between the presence of manganese or iron, whereas strong fluorescence spectra recorded at the European Synchrotron Radiation Facilities (Grenoble) revealed unambiguously the presence of only iron in
crystals. It was concluded that the Pce domain had a binuclear center containing iron, although the expected metal was zinc, present in the majority of the members of the metallo-β-lactamase superfamily.

It appeared then important to verify that the iron ions were present in the purified Pce protein in solution. The apo form of Pce was generated, and the protein was reconstituted by incubation with iron and calcium ions and PCho molecules. The Pce was tested for NP-PC hydrolysis in the presence or absence of iron, calcium, and PCho (Fig. 3). Only addition of 1 mM Fe(II) increased the efficiency of catalysis (4-fold) compared with the oxidized iron state (Fe(III)) or other metals, such as zinc and calcium (Fig. 3). Hydrolysis was doubled when reducing agents such as DTT and Tris(2-carboxyethyl)phosphine were added to the reaction mix (data not shown). It is thus probable that the binuclear center present in the Pce active site is involved in the catalytic reaction and that the reduced state is more favorable for enzymatic activity.

The PCho Binding Site—The structure of Pce was solved in complex with the ligand PCho. The major interaction between Pce and PCho occurs between the phosphate group of PCho and the binuclear center (Fig. 2B). Two of the oxygens of the PCho phosphate group interact directly with iron ions, leaving the third oxygen pointing away from the binding site (Fig. 2B). This phosphate group orientation would allow Pce to bind a substrate bearing an ester moiety linked to PCho. In the active site, residues Arg146, Asp114, and His253 stabilize PCho by the side chains of two iron ligands His112, Ser113, Asp114, and Trp148 and the backbone oxygen of Gly78 that accommodates the positively charged trimethyl-ammonium group of PCho (Fig. 2B). A cation-π interaction is observed between the choline and Trp148, a feature found in other choline-binding proteins (44). In addition, Trp205 and Trp284 face the active site, residues Arg146, Asp114, and His253 further stabilize PCHO binding (Fig. 2B).

A unique feature of the active site is the small cavity formed by the side chains of two iron ligands His112, Ser113, Asp114, and Trp148 and the backbone oxygen of Gly78 that accommodates the positively charged trimethyl-ammonium group of PCho (Fig. 2B).

Structural Features of the Active Site: Pce Is Related to Metallo-phosphatase Enzymes—The metal binding site is situated at a topological equivalent position to that observed in other members of the metallo-β-lactamase superfamily (Fig. 5) (45).
The Pce coordination spheres of the metal ions are close to octahedral and involve the His$_{110}$-His$_{112}$-Asn$_{208}$ triad at site 1; zinc-containing members GOX, RNaseZ, and FEZ-1 display three histidines (48) (Fig. 5). For this enzyme the proposed nucleophile is a hydroxide ion bound to the Fe$_3^{3+}$, the alternative mechanism, using the bridging hydroxide ion as nucleophile, is less probable (54). Based upon the structural similarities between the Pce and Pap active sites, a Pap-like $S_N2$-type catalytic mechanism can be proposed for Pce (Fig. 6). The Pcho ester initially binds to iron site 2 through one of the non-esterified oxygen atoms of the phosphate (step 1), which undergoes nucleophilic attack by a hydroxyl ion bound to iron site 1, inducing an inversion of configuration on the phosphorus atom. These events first generate a pentacoordinate intermediate (step 2) and subsequently the complex (step 3, structure observed) in which the reaction product is bound in the active site (Fig. 6). The enzyme is most probably regenerated by the binding of a water molecule to iron site 1, promoting the release of the product by ligand substitution. Evidence in favor of this hypothesis are the following. (i) The left side of the active site is more accessible to the substrate, supporting an initial interaction of Pcho with iron site 2. (ii) A water molecule (Wat11 in MolA and Wat216 in MolB), which makes two hydrogen bonds with Arg146 and Asp206, is in perfect support of an initial interaction of Pcho with iron site 2. (iii) A water molecule activated to a hydroxyl; the nature of the metal-ligated water molecule activated to a hydroxyl; the nature of the metal-activated nucleophile is still under debate. It has been established that Pap hydrolysis of phosphate esters occurs with inversion of stereochemistry of the phosphorus (53). For this enzyme the proposed nucleophile is a hydroxide ion bound to the Fe$_3^{3+}$, the alternative mechanism, using the bridging hydroxide ion as nucleophile, is less probable (54). Based upon the structural similarities between the Pce and Pap active sites, a Pap-like $S_N2$-type catalytic mechanism can be proposed for Pce (Fig. 6). The Pcho ester initially binds to iron site 2 through one of the non-esterified oxygen atoms of the phosphate (step 1), which undergoes nucleophilic attack by a hydroxyl ion bound to iron site 1, inducing an inversion of configuration on the phosphorus atom. These events first generate a pentacoordinate intermediate (step 2) and subsequently the complex (step 3, structure observed) in which the reaction product is bound in the active site (Fig. 6). The enzyme is most probably regenerated by the binding of a water molecule to iron site 1, promoting the release of the product by ligand substitution. Evidence in favor of this hypothesis are the following. (i) The left side of the active site is more accessible to the substrate, supporting an initial interaction of Pcho with iron site 2. (ii) A water molecule (Wat11 in MolA and Wat216 in MolB), which makes two hydrogen bonds with Arg146 and Asp206, is in perfect position to interact with one phosphate group oxygen in the pentacoordinate intermediate, and (iii) a conserved role for His$_{253}$ in the catalytic mechanism, which may give a proton to the leaving group. To experimentally verify the proposed reaction mechanism, Pce mutants were constructed and enzymatic assays were performed using $p$NP-PC as a substrate (Table II). When His$_{253}$ was mutated to Asn, the mutant showed much lower enzymatic activity (15% of the wild-type value), which was too low for...
For the kinetic assays performed with Pce wild-type and the mutant Asp, the substrate pNP-PC was used at concentrations ranging from 0.16 to 32.8 mM in the presence of 1 mM Fe(NH$_4$)$_2$(SO$_4$)$_2$, 5 mM DTT, and 0.1 mM CaCl$_2$. The relative activities of mutant proteins compared to the wild-type were measured in a reaction mix containing 0.2 mM substrate, 0.5 μg of each Pce protein, 1 mM Fe(NH$_4$)$_2$(SO$_4$)$_2$, 5 mM DTT, and 0.1 mM CaCl$_2$. Similar values for D114N and H253N mutant proteins were obtained with substrate concentrations of 32.8 mM.

(i) iron liganding, and (ii) stabilization and activation of the reduced state.

It is reasonable to assume that one of the iron ions must be in the reduced state.

Asp114 has two roles in the Pce structure: (i) iron liganding, and (ii) stabilization and activation of the reduced state.

The 10 repeats define four probable choline binding pockets lined by conserved hydrophobic residues: Trp383, Trp390, and Met439 in site 1; Trp403, Tyr410, and Tyr430 in site 2; Trp423, Trp430, and Tyr471 in site 3; and Trp443, Trp450, and Met461 in site 4.

This observation suggests that Pce cannot hydrolyze all PCho molecules on the teichoic acid polymers because of structural constraint. The binding of up to two teichoic acid chains, B and C, can be modeled along the CBD, in a way to schematically visualize the attachment of CBPE to the cell wall (Fig. 7). In conclusion, CBPE is a very interesting multifunctional enzyme: the same substrate (PCho on teichoic acids) is recognized by both Pce and CBD regions although the two binding site architectures are not related (even if aromatic residues are always required). Furthermore the functions of these domains are totally different: Pce hydrolyzes the PCho ester, while CBPE phosphorylates the teichoic acid chains.

### DISCUSSION

CBPs are pneumococcal cell wall-associated macromolecules that display activities ranging from adhesion to proteolysis of host proteins. Here, we report the structural and biochemical characterization of Pce, the catalytic domain of CBPE from *S. pneumoniae*.

The overall structural fold confirms that the Pce region of CBPE belongs to the metallo-lactamase superfamily but major structural and functional features render Pce unique among this group of proteins. First of all, Pce displays a di-iron phosphatase-like reaction mechanism. Iron ions in the Pce active site structure were identified by fluorescence scans on crystals,
and their signal was employed to solve the structure. Furthermore, the presence of iron ions in the structure was verified to ensure that the binding of iron atoms was not a crystal artifact, but de facto physiological. Indeed, solution reconstitution of the Pce native state from the apo form could be achieved by incubation with iron, calcium, and PCho molecules. However, because different cations can bind in the metal sites of metallo-β-lactamase-like proteins, we performed enzymatic assays. Under our experimental conditions, all the results reveal that iron ions are essential for the PCho hydrolysis activity of Pce (57).

Fig. 5. Structural analogies of the Pce active site with members of the metallo-β-lactamase superfamily and with purple acid phosphatase. Scheme of the coordination sphere of the binuclear center of Pce; metallo-β-lactamase, FEZ-1 (PDB code 1L9Y); rubredoxin:oxygen oxidoreductase, ROO (PDB code 1ESD); glyoxalase II, GOX (PDB code 1QH5); t-RNA maturase, RNase Z (PDB code 1Y44); purple acid phosphatase, Pap (PDB code 1UTE).

FIG. 6. Scheme of the proposed mechanism of catalysis for Pce. The PCho is represented in blue and the ester group in green.
The presence of iron ions and PCho molecules in the active site, as well as the nature and geometry of the coordination ligands, suggest that the catalytic properties of Pce are more closely related to that of metallo-phosphatase enzymes than to those of metallo-β-lactamase hydrolases. Based on this observation, we proposed a reaction mechanism that was supported by site-directed mutagenesis. The structure of the active site determined in this work corresponds to the oxidized state of the enzyme complexed to the reaction product. Because enzymatic regeneration must proceed by entry of a water molecule in metal site 1, the structure observed may correspond to an inactive enzyme. The reduced state of iron in site 1 must be essential in the catalysis process; this hypothesis is in accordance with the observed increased activity of the native form of Pce in the presence of reducing agents. However, the reduced/oxidized state of each iron ion remains to be elucidated, using, for example, the electron paramagnetic resonance method. Nevertheless, these observations open questions about the optimal physiological conditions for Pce function. For example, is Pce most efficient in an anaerobic environment (inside the host tissue) than in an aerobic environment (on nasopharyngeal epithelial cells)?

Kinetic parameters were determined for Pce wild-type and mutant enzymes, using the synthetic substrate pNP-PC. Even for the wild-type protein, the measured values of $k_{cat}/K_m$ are in the $10^{-3}$ s$^{-1}$M$^{-1}$ range, indicating that the protein harbors very low catalytic efficiency (36). This point raises the question of the relevance of the pNP-PC molecule as a substrate for Pce, although the use of this synthetic substrate has been of central importance for the identification of CBPE in pneumococcal cell extracts (34, 36).

The Pce active site harbors a structural characteristic not shared by any member of the metallo-β-lactamase superfamily, the presence of an elongated loop lining the top part of the active site. The orientation, interactions, and calcium stabilization of this loop suggest a role in the accessibility of substrate into the catalytic cavity of Pce. Asp$^{62}$ bridges both calcium ions, and although its mutation to Ala had no effect on enzymatic efficiency with pNP-PC as a substrate (neither with exogenous calcium added to the apoPce), this does not exclude the possibility that calcium may play a role in catalysis when native
substrate binds to the active site. In addition, the concentration of free calcium under physiological conditions may also influence the orientation/stabilization of the elongated loop and the active site accessibility.

CBPE has been shown to remove only 20% of the PCho residues from the cell wall teichoic acids (35). The limited activity of the enzyme was suggested as being attributed to the poor spatial accessibility of the PCho molecules; however, this low enzymatic efficiency reflects the catalytic results presented in this work using the synthetic substrate, indicating that in vitro, under aerobic conditions, Pce has low activity. Nevertheless, the virulence effect of CBPE may be the consequence of its enzymatic property, which influences the quantity of PCho (known to bind platelet-activating factor receptor on host cells) and may also regulate the presence of CBPs at the bacterial surface. However, results obtained by Gosink et al. (23) with a pneumococcal strain deleted in cbpE, i.e., decreased nasopharynx colonization and cell adherence, suggest the hypothesis of a direct effect of the CBPE protein in virulence processes. In addition, CBPE may function either bound to the bacterial surface or released in the extracellular medium. By catalyzing the cleavage of PCho molecules, CBPE may proceed to its own release by competition with teichoic acid-bound PCho residues.

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Crystal Structure of Phosphorylcholine Esterase Domain of the Virulence Factor Choline-binding Protein E from *Streptococcus pneumoniae*: NEW STRUCTURAL FEATURES AMONG THE METALLO-β-LACTAMASE SUPERFAMILY

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