Large-scale purification and characterization of recombinant Pseudomonas ceramidase: regulation by calcium

Bill X. Wu, Christopher F. Snook, Motohiro Tani, Erika E. Büellesbach, and Yusuf A. Hannun

Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, SC 29425

Abstract Ceramidases (CDases) hydrolyze ceramide to sphingosine (SPH) and fatty acid. Pseudomonas CDase (pCDase) is a homolog of mammalian neutral ceramidases and may play roles in disease pathogenesis. In this study, pCDase was cloned and expressed in Escherichia coli (E. coli). The expressed recombinant pCDase was solubilized by optimizing several factors, including culture medium, the concentration of isopropyl-β-thiogalactopyranoside (IPTG), temperature, and time of induction, which were identified to be critical for the optimal production of recombinant pCDase. The recombinant pCDase was purified using nickel-nitrilotriacetic acid affinity, phenyl-Sepharose, and Q-Sepharose column chromatography, which gave an overall yield of 0.45 mg/l purified protein of starting culture. The activity of the recombinant pCDase followed classical Michaelis-Menten kinetics, with optimum activity in the neutral pH range. Both the hydrolytic and the reverse activities of pCDase were stimulated by calcium with an affinity constant (Kₐ) of 1.5 μM. Kinetics studies showed that calcium caused a decrease of Kₐ and an increase in Vₘₐₓ of pCDase. Calcium and o-erythro-sphingosine caused significant changes in the near ultraviolet circular dichroism (CD) spectra and the changes were inhibited in the presence of EGTA. These results identify important interactions between calcium and pCDase, which may play an essential role in the interaction of pCDase and its substrate. — Wu, B. X., C. F. Snook, M. Tani, E. E. Büellesbach, and Y. A. Hannun. Large-scale purification and characterization of recombinant Pseudomonas ceramidase: regulation by calcium. J. Lipid Res. 2007. 48: 600–608.

Supplementary key words circular dichroism • sphingolipid • ceramide

Sphingolipids, initially identified as important structural components of cell membranes, are now also recognized as vital regulators and mediators of many biological responses (1, 2). Among these bioactive species of sphingolipids, ceramide, sphingosine (SPH), ceramide-1-phosphate, and sphingosine-1-phosphate (S1P) have been extensively studied and identified as essential bioactive molecules in mediating cellular signaling pathways, including cell growth arrest, differentiation, apoptosis, chemotaxis, and inflammation (3–7). The levels and turnover of these bioactive molecules are controlled by several enzymes that play essential roles in the regulation of sphingolipid-mediated pathways.

Ceramidases (CDase, EC3.5.1.23) catalyze the cleavage of the N-acyl linkage in ceramide to form SPH and free fatty acid. Therefore, ceramidases play important roles in the regulation of the substrate ceramide, the immediate product SPH, and the further downstream metabolite S1P. According to the catalytic pH optima and primary sequence, CDases have been classified into three different groups: acid CDases, alkaline CDases, and neutral CDases. Genetic analysis reveals that the three CDase families are derived from different ancestral genes (8). Among the CDases, acid CDase is a lysosomal enzyme and its mutations cause the human disorder Farber’s disease (9). Recently, several alkaline CDases were also cloned and characterized, including phytoCDase and dihydroCDase from yeast Saccharomyces cerevisiae (10, 11), a homolog of phytoCDase from human (12) and mouse alkaline ceramidase (13).

Neutral CDases (nCDases) have been cloned from many species, including, Pseudomonas aeruginosa (P. aeruginosa) (14), human (15), mouse (8), rat (16), Drosophila melanogaster (17), Dicytostelium discoideum (18), and zebrafish (19). Mammalian nCDases have also been purified to homogeneity and biochemically characterized from various tissues, e.g., mouse liver (20) and rat brain, kidney, and intestine (16, 21, 22). Mammalian nCDases appear to...
play roles in cell proliferation and apoptosis by regulating the levels of ceramide, SPH, and SIP. For example, studies showed that retinal degeneration in Drosophila mutants can be rescued by targeted expression of human neutral CDase (23). In mesangial cells, nCDase was up-regulated by platelet-derived growth factor (24). Another report showed that nCDase also play roles in mediating the effects of advanced glycation end-products on cell proliferation (25). Dysfunction of nCDase causes impaired vesicle fusion and trafficking in the Drosophila nervous system (26).

nCDase has also been identified and characterized in two Pseudomonas strains (14, 27). P. aeruginosa is a gram-negative pathogen that can cause severe infection in patients with sepsis, cystic fibrosis, burns, or immunocompromised conditions. During Pseudomonas infection, ceramide has been proposed to play essential roles in host defense responses by reorganizing membrane rafts for internalization of the bacterium and in initiating immune signaling (28). Other studies provided evidence that the Pseudomonas ceramidase (pCDase) might play roles in hydrolysis of ceramide in skin in atopic dermatitis (29, 30).

Activity assays have shown that nCDase can catalyze both the hydrolysis of ceramide and its reverse reaction, the synthesis of ceramide, from SPH and fatty acid (31–33). The enzyme is regulated by anionic phospholipids and by calcium. However, the mechanism of calcium activation is unclear. Moreover, although extensive efforts have been made to investigate the biochemical properties of nCDase, no structural information is available.

In this study, recombinant pCDase was expressed and solubilized by optimizing the expression conditions. The recombinant protein was purified and characterized. Importantly, calcium was shown to activate the enzyme with an affinity constant \((K_a)\) of 1.5 \(\mu\)M, suggesting a relatively high affinity of interaction. By analyzing the circular dichroism (CD) spectrum of pCDase, the secondary structure was predicted, and near UV CD spectrum analysis also revealed significant interaction between calcium and pCDase. Therefore, this study not only provides knowledge for the regulation and biochemical properties of pCDase but also demonstrates that CD spectroscopy is a useful tool for the analysis of interactions between the enzyme, its substrates, and calcium activator.

**EXPERIMENTAL PROCEDURES**

**Materials**

All biochemicals were obtained from Sigma (St. Louis, MI) unless otherwise stated. \(\alpha\)-erythro-C\(_{12}\)-NBD-ceramide and \(\alpha\)-erythro-sphingosine were kindly provided by the Lipidomics Core Facility at the Medical University of South Carolina. NBD-C\(_{12}\) fatty acid was obtained from Molecular Probes (Eugene, OR). Anti-His\(_6\) monoclonal antibody was obtained from Novagen (Madison, WI).

**Cloning the pCDase**

The DNA sequence encoding the P. aeruginosa PA01 ceramidase was identified using BLAST software (http://www.ncbi.nlm.nih.gov/BLAST) against the P. aeruginosa PA01 genome sequence (NC002516), using CDase cDNA sequence identified from P. aeruginosa strain AN17 (AB028646) (14). A Champion™ pET directional TOPO expression kit (Invitrogen, Carlsbad, CA) was used to clone and express the pCDase. Two oligonucleotides, a forward primer (5’-CACCATGTCAGTCCCGCATTCCCGGC-3’) and a reverse primer (5’-CTTGTGGCTAGACCGCTTATGATGAT-3’), were synthesized. Using the above primers and Pseudomonas PA01 genomic DNA as a template, the full-length coding sequence of pCDase (including the N-terminal signal peptide sequence) was amplified by Pfu-Utra High Fidelity DNA polymerase and then cloned into pET101/D-TOPO vector (Invitrogen), directly followed by transformation of the construct into E. coli BL21 Star™(DE3) strain according to the manufacturer’s instruction.

**Expression of pCDase**

For optimization of expression and induction conditions, the E. coli pCDase-BL21 was first grown in Luria-Bertani (LB) medium supplemented with 50 \(\mu\)g/ml ampicillin overnight, then transferred to M9 minimum or LB medium to optical density 600 of 0.1, and cells were grown to optical density 600 of about 0.5–0.8 (mid log) at 37°C. Induction conditions for expression of pCDase were optimized using different concentrations (0.1–1 mM) of isopropyl-beta-thiogalactopyranoside (IPTG), or different periods (3, 6 and 24 h) at different temperatures.

To detect the quantity and solubility of the expressed pCDase, the pCDase-BL21 E. coli. cells were fractionated. The culture was first centrifuged at 3000 \(\times\) g for 15 min to separate culture medium and cells. The pCDase-BL21 cell pellet was lysed by sonication and the unlysed cells were pelleted by centrifugation at 180 \(\times\) g for 15 min. The supernatant was then centrifuged at 100,000 \(\times\) g for 30 min to separate total membrane (insoluble) from soluble proteins. The supernatant was centrifuged again to remove any contaminant. The pellets were washed twice and then solubilized in 1% (v/w) Triton-X100. Equal amounts of protein from each fraction were subjected to SDS-PAGE and Western blot analysis using antibody specific to the His-tag as described previously (34).

After optimization, the induction condition for pCDase expression was finalized using 0.5 mM IPTG to pCDase-BL21 in M9 minimum medium for 24 h at 30°C before harvest.

**Purification of recombinant Pseudomonas nCDase**

Protein concentration was determined by the BCA method (Pierce, Rockford, IL) using BSA as a standard. Protein purification was performed using a Pharmacia Acta-FPLC and columns purchased from GE health care. Cells were lysed by a combination of lysozyme treatment and sonication using a Model 500 sonicator (Roche, Switzerland) and incubated at 37°C for 1 h with gentle stirring. The lysate was then subjected to 10 s sonication and the unlysed cells were pelleted by centrifugation at 180 \(\times\) g for 15 min. The supernatant was then centrifuged at 100,000 \(\times\) g for 30 min to separate total membrane (insoluble) from soluble proteins. The supernatant was centrifuged again to remove any contaminant. The pellets were washed twice and then solubilized in 1% (v/w) Triton-X100. Equal amounts of protein from each fraction were subjected to SDS-PAGE and Western blot analysis using antibody specific to the His-tag as described previously (34).

After optimization, the induction condition for pCDase expression was finalized using 0.5 mM IPTG to pCDase-BL21 in M9 minimum medium for 24 h at 30°C before harvest.
CDase enzyme assays and biochemical characterization

Enzyme activity was measured using detergent/lipid mixed micelles with \( \text{d-erythro}\text{-C}_{12}\text{-NBD-ceramide} \) as a substrate at a concentration of 50 \( \mu \text{M} \) or 1.08 mol% in a 50 mM (pH 7.1) Tris buffer containing 1 mM CaCl\(_2\), 0.3% (w/v) Triton X-100 final concentration, with a total volume of 100 \( \mu \text{L} \). The enzymatic reaction was started by adding 1 \( \mu \text{g} \) of purified enzyme (unless otherwise stated). For the pH optimum determination, the substrate was dissolved in the following buffers: (pH 3.7–5.5) 50 mM acetate buffer, (pH 5.7–6.7) 50 mM MES buffer, (pH 6.3–7.3) 50 mM phosphate buffer, (pH 7.1–9.0) 50 mM Tris buffer, and (pH 8–10) 50 mM glycine-NaOH buffer. The enzyme reaction was continued for 30 min at 37°C, and it was stopped by addition of chloroform/methanol (1:1). The organic phase was spotted onto TLC plates and analyzed as described previously (34).

To determine the calcium effects on the recombinant CDase activity, 0.1 to 1 mM CaCl\(_2\) were included in the 100 mM Tris buffer (pH 7.1) buffer with 1 mM EGTA. The free Ca\(^{2+}\) concentrations were calculated using WEBMAXC software (http://www.stanford.edu/~cpatton/webmaxc/webmaxcE.htm) (35). The kinetic parameters \( K_{in} \) and \( V_{max} \) were analyzed by Enzyme Kinetics software (SPSS).

The reverse CDase activity assays were performed as described (31) with modifications. Briefly, the substrates, 0.25 nmol of \([\text{H}]\text{palmitic acid, 9.75 nmol of unlabeled palmitic acid, and 10 nmol d-erythro-sphingosine were dried and then resuspended in 100} \) \( \mu \text{L} \) of reaction buffer[100 mM Tris buffer (pH 7.1) 0.2% (w/v) Triton X-100]; 1 ng recombinant CDase was used for each reverse reaction, and the enzymatic reaction was conducted at 37°C for 2 h. The lipids were extracted and loaded on TLC plates as described above. The produced ceramide was visualized by autoradiography. The ceramide band and total remaining lipids were scraped separately for liquid scintillation counting. The percentage of ceramide synthesis for the reaction was calculated by comparing \([\text{H}]\text{ceramide band with total lipid.}

CD measurements

The protein concentration of CDase stock was determined by ultraviolet (UV) spectroscopy using an Olis Cary-15 spectrometer conversion in combination with a specific absorbance coefficient of 1.18 ng\(^{-1}\text{cm}^{-1}\) calculated by GCG peptidesort program (Genetics Computer Group, Inc, Madison, WI.). CD spectra were recorded using a Jasco J710 spectropolarimeter at ambient temperature at a resolution of 0.2 nm. All spectra were corrected for background by the subtraction of the buffer blank.

Far UV CD spectroscopy (190–250 nm) was measured in a cell of 0.1 cm pathlength. The \( \text{Pseudomonas} \) CDase solution was diluted to 0.28 mg/mL (~3.9 \( \mu \text{M} \)) in buffer containing 50 mM Tris (pH 7.4) and 40 mM octyl-\( \beta \)-glucoside. To test the effects of \( \text{d-erythro-sphingosine or calcium on CD spectra, 120} \) \( \mu \text{M d-erythro-sphingosine, 0.5 mM CaCl}_2\), or EGTA, was added to the buffer system, respectively. Ten spectra were averaged for each derivate. For spectra containing ultraviolet absorbing additives (i.e., \( \text{d-erythro-sphingosine and EGTA} \)), spectra were reported in the range of 197–250 nM, mean residue ellipticity \(((\theta)\text{mrw})\) was calculated and helix content was estimated using the assumption that \(((\theta)\text{mrw})\text{100}\) for 100% helix is 40,000 (36).

For near UV CD spectroscopy (250–320 nm), a 1 cm pathlength cell was used, and forty spectra were averaged. Recombinant CDase (0.28 mg/ml) was dissolved in 50 mM Tris (pH 7.4), 4 mM CHAPS, and the circular dichroism measured in the presence and absence of 120 \( \mu \text{M d-erythro-sphingosine or N-oleoylsphinganine (NOE)} \), in the presence of 0.5 mM CaCl\(_2\) or EGTA. Spectra are displayed as molar circular dichroism (\( \Delta \varepsilon \)).

RESULTS

Optimization of the pCDase expression

In order to examine structural and biochemical properties of pCDase, we established an expression system for large-scale purification. The full-length DNA coding sequence, encoding the pCDase, was cloned into pET101/D-TOPO vector under the control of the \( \text{lac} \) promoter. A His\(_6\) tag was added to the C terminus to facilitate subsequent detection and isolation. The pCDase-BL21 \( \text{E. coli} \) cells were first grown in LB medium and induced with 1 mM IPTG for 6 h. After fractionation of cell lysate, the majority of expressed pCDase was found in the insoluble fraction (the 100,000 \( \times \) g pellet) using Western blot analysis (Fig. 1A). However, CDase activity was mainly in the soluble fraction (data not shown), suggesting that the majority of expressed pCDase was insoluble and largely inactive under these conditions. Thus, further optimization was necessary to increase the percentage of soluble protein and minimize the generation of inactive protein. First, different media were tried. As shown in Fig. 1A, expression induced in M9 minimum medium showed an approximate 3-fold increase of soluble protein when compared with expression in LB medium. Thus, M9 minimum medium was selected for the remaining optimization procedures. The optimum concentration of IPTG for the solubility of expressed pCDase was established next. As shown in Fig. 1B, the percentage of soluble pCDase increased with decreasing of IPTG concentration from 1 to 0.1 mM. Because 0.1 mM IPTG gave low expression, a concentration of 0.3 mM IPTG was employed for further optimization of induction time and temperature. Interestingly, when the induction temperature was decreased to 30°C for 24 h, the majority of recombinant pCDase became soluble while only trace amounts of pCDase were detected in the 100,000 \( \times \) g pellet (Fig. 1C). Because the pCDase has been purified from medium as a secreted protein (32), we also tested for the recombinant pCDase in the medium. However, only trace amounts of pCDase were detected. Thus, the culture medium was not suitable for the purification of recombinant CDase under tested conditions. Therefore, by optimizing the culture and induction conditions, the expression of soluble and active protein was maximized, providing the basis for further purification and characterization.
Purification of recombinant pCDase

The pCDase-BL21 cell pellet was lysed by a combination of lysozyme treatment and sonication. After precipitation of nucleic acids using protamine sulfate, the supernatant was subjected to three chromatographic purification steps (Fig. 2A). Thus, soluble fractions of crude cell-free extracts were first subjected to Ni affinity chromatography, followed by phenyl-sepharose chromatography, and finally by Q-sepharose high performance chromatography. Although the specific activity was not markedly increased by the latter steps (phenyl-sepharose and Q-FF columns), several impurities were removed as indicated by SDS-PAGE analysis (Fig. 2B). The purified pCDase migrated at about 70 kDa, matching the expected molecular mass as deduced from the cDNA sequence and previous reports on recombinant proteins (14, 27, 32). As judged by SDS-PAGE densitometry, the enzyme obtained was 98% pure and had a high specific activity (2.7 μmol/min/mg), significantly higher than the purified recombinant pCDase of PAO1 reported previously (27). The recombinant pCDase was purified to near homogeneity with approximately 31% recovery of activity, summarized in Table 1 and Fig. 2B.

Characterization of the expressed pCDase

The biochemical properties of purified recombinant pCDase were examined. As shown in Fig. 3A, the recombinant pCDase displayed a broad pH range with an optimum pH activity in the neutral range. The pH optimum is similar to those of mammalian nCDases. Previous studies have suggested that pCDase is a calcium-dependent enzyme (14, 32). However, the relevant concentrations of calcium were not determined. Therefore, to determine the optimal concentration of free calcium for pCDase activity, free calcium concentrations were varied from 0.02 to 18.5 μM using EGTA/calcium buffers, and the concentrations of free calcium were calculated using MAXCHELATOR, a software established to determine free metal concentrations in the presence of chelators (http://www.stanford.edu/~cpatton/maxc.html) (35). As shown in Fig. 3B, pCDase activity increased with calcium concentrations with an apparent Ka of 1.5 ± 0.4 μM. The reverse CDase activity of pCDase was also activated by calcium with a similar Ka (Fig. 3D). To exclude the possibility of His-tag interference in the calcium effects on pCDase, we also partially purified native pCDase from

Fig. 1. Optimization of conditions for expression of Pseudomonas ceramidase (pCDase). BL21-pCDase cells were grown in M9 minimum or Luria-Bertani (LB) media and then induced with isopropyl-beta-thiogalactopyranoside (IPTG). Cell lysates were fractionated, and then 0.1% of each fraction as well as the culture medium were loaded to SDS-PAGE and analyzed by Western blot using His-tag antibody, as described in Experimental Procedures. A: Effects of LB or M9 minimal medium on solubility of recombinant pCDase. Cells were grown in LB or M9 minimal medium at 37°C and induced with 1 mM IPTG for 6 hr. B: IPTG effects on pCDase solubility. Ceramidase (CDase) expression was induced by different concentrations of IPTG for 6 h in M9 minimal medium at 37°C. C: Time and temperature effects on pCDase expression. Recombinant pCDase was induced by 0.3 mM IPTG for different periods in M9 minimal medium at 30°C or 37°C. M, culture medium; P, 100,000 × g pellet; S, 100,000 × g supernatant; W, whole lysate.
200 ml culture medium of *P. aeruginosa* PAO1 using a diethylaminoethanol column as described previously (32), and the $K_s$ for calcium were measured. The results (supplementary Fig. 1) showed that the native pCDase has the same $K_s$ (1.5 μM) as the recombinant His-tagged pCDase, thus demonstrating that the His-tag did not interfere with the calcium effects on pCDase.

To determine the kinetic mechanisms of activation of pCDase by calcium, the effects of calcium on kinetic parameters were investigated at three different concentrations of free calcium (0.26, 0.7, and 18.5 μM). Interestingly, increasing calcium from 0.26 to 18.5 μM caused a decrease in the $K_m$ of around 50%, and the $V_{max}$ increased from 2.1 to 8.1 mol/min/mg (Fig. 3C). This result suggests that calcium activates the pCDase in a mixed mechanism by enhancing both the substrate-enzyme affinity and catalytic capability.

### Table 1. Purification of recombinant pCDase

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein</th>
<th>Activity</th>
<th>Specific activity</th>
<th>Recovery</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell lysates</td>
<td>2776</td>
<td>30.5</td>
<td>0.011</td>
<td>100.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Supernatant</td>
<td>1250</td>
<td>23.7</td>
<td>0.019</td>
<td>77.8</td>
<td>1.3</td>
</tr>
<tr>
<td>Protamine sulfate supernatant</td>
<td>974</td>
<td>19.5</td>
<td>0.020</td>
<td>63.8</td>
<td>1.8</td>
</tr>
<tr>
<td>Nickel chelation column</td>
<td>7.5</td>
<td>14.4</td>
<td>1.9</td>
<td>47.1</td>
<td>174.4</td>
</tr>
<tr>
<td>Phenyl-sepharose</td>
<td>4.3</td>
<td>10.8</td>
<td>2.5</td>
<td>35.2</td>
<td>227.4</td>
</tr>
<tr>
<td>Q-sepharose</td>
<td>3.6</td>
<td>9.6</td>
<td>2.7</td>
<td>31.3</td>
<td>241.6</td>
</tr>
</tbody>
</table>

CDase was purified from eight liters of culture as described in Experimental Procedures. One enzyme unit was defined as the amount capable of hydrolyzing 1 μmol CI2-NBD-ceramide/min at 37°C using $\beta$-erythrosphingosine as a substrate at a concentration of 50 μM or 1.08 mol% in a 50 mM (pH 7.1) Tris buffer contained 1 mM CaCl$_2$, 0.5% (w/v) Triton X-100 final concentration, with a total volume of 100 μl.

### Far UV CD analysis

To acquire structural information and determine the conformational effects of calcium and substrates on the purified pCDase, CD spectroscopy was conducted (Fig. 4). The value of pCDase [9] mrw222 was about 6000 deg cm$^2$/dmol$^{-1}$ corresponding to a 15% $\alpha$ helix content (36). A secondary structure prediction from the PSIPRED program (37) gave a similar prediction with 20% $\alpha$-helices. These results suggest that the helical content of pCDase is likely in the range of 15–20%.

### Calcium and SPH effects on CD spectra of pCDase

Because calcium activated pCDase, the effects of calcium on pCDase were investigated by CD spectroscopy. Initially, studies were performed in the far UV region; however, neither calcium nor EGTA affected the far UV spectra (Fig. 4A). The effects of the product of the reaction, $\beta$-erythrosphingosine, which also serves as a substrate for the reverse reaction of the enzyme, were examined. As sphingolipids need to be solubilized for optimal delivery, a detergent needed to be chosen that dissolved sphingolipids but did not absorb UV light to allow for CD measurements. Specifically, triton X-100, used for activity studies, has a strong absorption band at 280 nm, and was therefore replaced by octyl-$\beta$-glucoside for far UV measurements and by CHAPS for near UV measurements. In either octyl-$\beta$-glucoside or CHAPS, pCDase retained full enzymatic activity while the background UV absorption was low. $\beta$-erythrosphingosine in the presence and absence of calcium did not affect the CD in the far UV (Fig. 4B).

Because far UV CD spectroscopy is primarily influenced by secondary structure, these results suggest that interactions between substrates or calcium with pCDase do not result in major changes in the secondary structure of pCDase.

On the other hand, changes in conformation may produce a difference in near UV CD spectra (320–250 nm), which are sensitive to changes in the tertiary structure of proteins (38). When the enzyme was incubated with calcium, it was found that calcium alone induced a clear change of the CD spectrum, affecting mostly the range from 250 to 265 nm (Fig. 4A, B). Therefore, calcium appears to modulate the overall conformation of pCDase but not its secondary structure.

In the presence of calcium, $\beta$-erythrosphingosine caused further significant changes throughout the whole UV region (Fig. 5A, B). Comparing with the pCDase near UV spectra in the presence of calcium, maximum changes were observed at 250–260 nm, while another broader region (270–310 nm) changed significantly with the maximum change at 288 nm (Fig. 5D). Moreover, the SPH effects on the pCDase UV CD spectra were significantly inhibited by the presence of EGTA (Fig. 5E, F), demonstrating a requirement for the free calcium. Taken together, these results show that calcium and SPH interact with the enzyme and result in significant changes in its tertiary structure.

Furthermore, we employed NOE, a ceramide analog and inhibitor of ceramidases, to determine its effects on near UV CD spectra of pCDase. Interestingly, NOE also induced
significant changes of near UV CD spectra in the presence of calcium that were inhibited by EGTA (Fig. 5G, H). These results suggest that NOE may interact with pCDase directly.

DISCUSSION

In this study, pCDase was expressed, purified, and characterized. The induction conditions of the expression system were optimized, and the optimization resulted in the majority of the expressed pCDase in soluble form. The recombinant pCDase was also purified to apparent homogeneity using a three-column protocol. Calcium action on the enzyme and the mechanism for calcium activation of pCDase were studied using kinetic analysis and CD spectroscopy. These results revealed that calcium interacted with pCDase as a cofactor, and this interaction is essential for the activation of pCDase.

The current study identified a specific and relatively high-affinity interaction between neutral ceramidase and calcium. It has been shown that calcium enhances the activity of pCDase (14, 32). The current study focused on determining the mechanism of activation mechanism of pCDase by calcium. Analyzing the two activities of the recombinant enzyme, hydrolytic and reverse CDase activities, revealed a similar $K_a$ of about 1.5 μM of the enzyme for free calcium (Fig. 3B, D). These results excluded the possibility that the activation occurs via calcium substrate interaction because that would have resulted in different $K_a$s for these two types of activity assays using different substrates. Further kinetics analysis showed that free Ca$^{2+}$ decreased the $K_m$ of the enzyme toward its substrate ceramide (Fig. 3C), suggesting that calcium increases the affinity of the enzyme toward its substrate, probably through conformational changes. Interestingly, calcium also increased the $V_{\text{max}}$ of pCDase toward its ceramide substrate,
suggesting calcium can also modify the catalytic activity of the enzyme, suggesting effects of calcium on conformation of the enzyme and its optimal interaction with substrate.

These suggestions on conformational effects of calcium on the enzyme were probed directly using CD spectroscopy (Fig. 5). CD spectroscopy is an important tool to detect protein-protein and protein-ligand interactions, which typically do not produce a detectable difference in far UV CD spectroscopy but may produce a difference in near UV CD spectra. As shown in Fig. 5, calcium induced a significant spectral change around 253 nm. Further, in the presence of calcium, SPH caused significant changes throughout the near UV spectrum, suggesting additional conformational changes of pCDase upon interaction with SPH (substrate for the reverse reaction). The broad range implies that the chiral environment of multiple aromatic side chains must change upon substrate binding. Because pCDase contains 29 phenylalanines, 9 tryptophans, and 26 tyrosines, CD is not suitable to dissect the affected aromatic groups. For more precise measurement of substrate-induced conformational changes, X-ray crystallography would be the method of choice.

The strong requirement for activation of pCDase by calcium, and the high affinity of the interaction, raise possible pathophysiologic implications through regulation of Pseudomonas infections. Previous studies suggest that pCDase might play a role in skin diseases (29, 30). Interestingly, while Pseudomonas can cause serious infections in wounded skin, it cannot colonize or survive on healthy dry skin (39). In damaged skin, Pseudomonas is exposed to high levels of calcium in the extracellular fluid, which is unavailable on the surface of healthy skin. It is possible that this high level of extracellular calcium activates the secreted pCDase, which then hydrolyzes skin ceramides and results in exacerbation of dermatitis. Further, ceramide plays essential roles in host defense of Pseudomonas infections. After infection of mice with Pseudomonas, interruptions of generation of ceramide-enriched raft platform was shown to result in unabated inflammatory responses and septic death of mice (28). Thus, during infection with Pseudomonas in cystic fibrosis patients, extracellular calcium may activate pCDase, which could then interfere with the host defenses by degrading ceramide, possibly in rafts in outer plasma membrane of the airway epithelium.

Moreover, the high affinity of activation of pCDase by calcium with a $K_a$ of around 1.5 $\mu$M raises the intriguing possibility that pCDase may be involved in the intracellular phase of Pseudomonas infection. While resting intracellular calcium is usually around 0.1 $\mu$M, many stimuli and agonists can rapidly raise the concentration to over 1 $\mu$M. This could then result in activation of pCDase which would decrease ceramide levels and increase those of SPH and S1P. These possibilities are worthy of further investigation.

A major goal for purification of recombinant pCDase in large amounts was to begin structural studies on the enzyme, as there is a significant paucity of structures of enzymes of lipid metabolism and other hydrophobic proteins. Previous studies have reported the purifications of recombinant CDases from different P. aeruginosa strains (14, 27). While the purification of pCDase from the growth medium most likely resulted in an active enzyme, the amount was low (27). The purification of refolded pCDase from inclusion bodies produced large amounts of protein, but the contamination of incorrectly folded protein could not be excluded and also the specific activity of the purified pCDase was low (27). In another study, CDase from P. aeruginosa strain AN17 was expressed and purified, but the yield was not specified (14). In our study, in order to
detect the expressed pCDase and facilitate the purification process, a C-terminal His-tag was added to the recombinant protein. As shown in Fig. 1, by optimizing the culture and induction conditions including medium, induction time, and concentration of IPTG, we successfully minimized the production of insoluble pCDase, providing the basis for the CD spectroscopic analysis. This approach to large-scale purification should also enable future crystallization studies, which necessitate large amounts of high quality purified protein.

In conclusion, although far UV CD spectrum analysis was previously employed in the study of secondary structures of sphingomyelinases from Loxosceles venom (40), this study is the first report using CD spectra to explore...
enzyme-sphingolipid interactions, raising the possibility that CD spectroscopy could emerge as a valuable tool for studies of sphingolipid enzymes.

This work was supported by National Institutes of Health Grant CA-87584. The authors would like to thank Drs. Chiara Luberto and Fernando Alvarez-Vasquez, the DNA Sequencing Facility, and the Lipidomics Core at the Medical University of South Carolina for expert assistance.

REFERENCES


