**INTRODUCTION**

In late 90s, a superfamily of proteins annotated as metal-dependent phosphohydrolase has been characterized as HD superfamily. As His and Asp (H...HD...D) were the highly conserved metal-coordinating residues in these proteins, phosphohydrolase activity was suggested to depend on coordination with divalent cations (Aravind and Koonin, 1998). The substrates of these proteins for nucleotidase and phosphodiesterase activities include variety of biomolecules with phosphoester bonds. Activity of HD superfamily proteins involves nucleotide metabolism, signal transduction, and other specialized functions in all the three domains of life (Proudfoot et al., 2004; Ryan et al., 2006; Worsdorfer et al., 2013). Although HD domain superfamily presently contains thousands of members, a limited number of proteins have been characterized on structural and functional basis. Besides its industrial applications, *Corynebacterium glutamicum* is capable of metabolizing variety of organic compounds and a clear link between aromatic catabolism and gluconeogenesis via fructose-1,6-biphosphate has been recognized (Qi et al., 2007). Also, three proteins, NCgl0524, NCgl0525, and NCgl0527 increased their abundance when 2-dimensional gels were compared after *C. glutamicum* growth on 4-cresol. NCgl0525 and NCgl0527 were identified to contribute towards 4-cresol metabolism (Qi et al., 2007). Recently, *cre* operon (ncg0521 to ncg0531) involved in 4-cresol metabolism has been genetically characterized, which revealed that 4-cresol is metabolized via 4-hydroxybenzoate and protocatechuic acid as intermediate metabolites and NCgl0524 (CreD) was involved in conversion of 4-cresol into 4-hydroxybenzyl alcohol (Li et al., 2014). CreD was characterized as Mg$^{2+}$-dependent phosphohydrolase with conserved HD domain. In the present report metal dependence before and after chelation, and thiol inhibition on phosphohydrolase activity of CreD were studied. In addition, kinetic parameters were determined and analysis of sequence data and phylogeny was performed.

**MATERIAL AND METHODS**

Protein expression, purification and molecular mass determination

CreD was expressed and initially purified as described (Li et al., 2014) and then purified by Superdex$^{	ext{TM}}$ 200 gel chromatography with Tris-HCl buffer (20 mM, pH 7.5) controlled by fast protein liquid chromatography system (AKTA, GE Healthcare, UK). After ultra filtration, concentration was determined by Bradford assay according to manufacturer’s instructions (Bio-Rad, Hercules, CA). Native molecular mass was estimated by size-exclusion chromatography on Superdex 200 GL column (GE Healthcare, UK) eluted with 50 mM Tris-HCl (pH 7.6) containing 100 mM NaCl at a flow rate of 0.4 ml min$^{-1}$.

**Metal dependence of phosphohydrolase activity of CreD**

Phosphohydrolase activity was determined with 4-nitrophosphophosphate (4-NPP) as substrate (Proudfoot et al., 2004) with divalent cations (Ba$^{2+}$, Cu$^{2+}$, Cu$^{+}$, Mg$^{2+}$, Mn$^{2+}$, NC$^{2+}$, and Zn$^{2+}$). The reaction mixture (50 mM of CHES-K buffer (pH 8.5), 5 mM cation, 4 mM 4-NPP, and 1 µg CreD) was incubated for 10 min before recording $A_{410}$, $\Delta$Amax, of 4-nitrophosphophosphate product. One enzyme unit was defined as amount of CreD liberating 1 mM of 4-nitrophosphate per min. Positive control (with CIP) and negative control (without CreD) were included and all experiments were conducted in triplicate. Non-iron-selective chelator, EDTA, was used to chelate divalent cations from CreD. The reaction mixture contained 50 mM CHES-K buffer (pH 8.5), 1.25–8.75 µM EDTA (pH 8.5), and 1 µg CreD. The reaction mixture was incubated at 37 °C for 10 min before addition of 4-NPP (4 mM) and further incubated at 37 °C for 10–20 min before recording $A_{410}$. After chelation with EDTA (6.25 mM), metal-dependence was determined as mentioned above. After incubation for 20 min at 37 °C, cation (5 mM) was added and reaction mixture was incubated at 37 °C for 10–20 min. Finally, $A_{410}$ was recorded. Both positive and negative controls were run in parallel.

**Effect of thiol (-SH) inhibition**

The reaction mixture (200 µl) contained 50 mM CHES-K buffer (pH 8.5), 5 mM Mg$^{2+}$, 1 µg CreD and 0.5–10 mM of various -SH inhibitors (dithiothreitol, iodoacetic acid, iodoacetamide and N-ethylmaleimide). The reaction mixture was incubated at 37 °C for 10 min before addition of 4-NPP (4 mM) and further incubated for 10–20 min before recording $A_{410}$. Both positive and negative controls were included in each set of experiments.

**Kinetic parameters**

Kinetic parameters, half-saturation constant or Michaelis constant ($K_m$), maximum reaction rate ($V_{max}$), turnover number ($k_{cat}$), and specificity constant ($k_{cat}/K_m$) were determined from Lineweaver-Burk plot. Reaction was carried out in 50 mM CHES-K buffer (pH 8.5) with 0.5–20 mM of 4-NPP at 37 °C as described. All reaction rates were linear during the course of reaction.
Hydrolase assay with deoxyribonucleotides (dNTPs) in vitro

Reaction mixture (160 μl) contained 50 mM CHES-K buffer (pH 8.5), 1 mM substrate (dNTP), and 0.1–1.0 μg CreD. After incubation for 20–30 min at 37 °C, the reaction was terminated by the addition of 40 μl malachite green reagent (Baykov et al., 1988), and after 5 min production of inorganic phosphate (Pi) was measured as A660 (Proudfoot et al., 2004). One unit of activity was defined as 1 μM of Pi produced per min.

Analysis of sequence data and phylogeny

CreD (NCgi0524) sequence (Accession No. NP599785) was obtained from GenBank. Sequence comparison and similarity search was performed using BLAST (http://www.ncbi.nlm.nih.gov). Pairwise and multiple sequence alignments were made with CLUSTAL W (Ayjar, 2000). Phylogenetic analysis was conducted using neighbor-joining method based on 1000 replications employing MEGA4 (Tamura et al., 2007).

RESULTS AND DISCUSSION

Subunit molecular mass of 27 kDa and native molecular mass of 54 kDa suggested that the biological unit is a dimer. This result is in accordance with reports on crystal structure and biochemical characterization of HD domain superfamily proteins. For instance, YhrF of E. coli is biologically active as a dimer (Zimmerman et al., 2008), dNTPase of Enterococcus faeicalis and human PDE4 (Hui et al., 2003) are biologically active as homotetramer, while dNTPase of Thermus thermophilus forms a homohexamer as a double ring of trimers (Kondo et al., 2007).

Phosphohydrolase activity profile of CreD with divalent cations is shown in figure 1A. Highest activity was achieved with Ba²⁺, Ca²⁺ and Mg²⁺ (6.78, 6.45, and 6.78 μM min⁻¹ mg⁻¹, respectively) while Cu²⁺, Mn²⁺, Ni²⁺, and Zn²⁺ did not support activity. Apparently, activity was still high in the absence of any cation that indicates coordination of some divalent cation with protein in its natural form when expressed in cells. Activity profile after chelation with EDTA is shown in figure 2. As indicated, activity gradually decreased with increasing EDTA concentration till 6.25 μM where there is no further decrease. After chelation, metal dependence of CreD is shown in figure 1B. Maximum phosphohydrolase activity (7.12 μM min⁻¹ mg⁻¹) was achieved with Mg²⁺. Now activity was significantly lower with same concentrations of Ba²⁺, Ca²⁺, and Co²⁺, which was considerably high without chelation (Figure 1A). No considerable activity was observed with Zn²⁺ and in the absence of any cation. Phosphatase activity of SurE and YjG of E. coli with 4-NPP is also Mg²⁺-dependent (Proudfoot et al., 2004). However, these results are different with those reported for YfhK and RNA-NT of E. coli, and human PDE4 where maximum activity was achieved with Co²⁺, Ni²⁺, and Zn²⁺, respectively (Proudfoot et al., 2004; Yakunin et al., 2004; Xu et al., 2000). Contrary to Kondo et al. (2007), no dNTPase activity was observed.

Cysteine residues were located at five different positions in CreD sequence (Cys²¹, Cys²³, Cys²⁵, Cys³⁸ and Cys⁴⁸), which contain free thiol (–SH) groups. These groups might form disulfide bonds that stabilize the overall structure of protein, which in turn co-ordinates with divalent cations to confer activity. Alkylation of these groups might produce certain structural changes that resulted in partial or complete loss of activity. Figure 3 indicates that thiol groups attached to Cys residues have significant contribution towards hydrolase activity of CreD. Iodoacetamide significantly inhibited phosphohydrolase activity (0.72 μM min⁻¹ mg⁻¹ at 1 mM concentration) while there was no apparent effect with 10 mM dithiothreitol. At 10 mM concentration, activity was fairly inhibited by N-ethylmaleimide (0.76 μM min⁻¹ mg⁻¹) whereas only partially with iodoacetamide (3.41 μM min⁻¹ mg⁻¹).

A plot of 1/[S] vs 1/V was a straight line (Lineweaver-Burk plot), where [S] and V were substrate concentration (μg μl⁻¹) and reaction rate (μM min⁻¹ mg⁻¹), respectively. The values of kinetic parameters Kₘ and Vₘₐₓ as indicated by the plot were 0.35 mM and 16.23 μM min⁻¹ mg⁻¹, respectively. The value of Kₘ placed CreD into the “high affinity” (millimole range) group of phosphohydrolases. Enzyme having high Kₘ requires a higher concentration of substrate to achieve Vₘₐₓ. Under normal conditions, enzyme activity varies with substrate concentration, which consequently controls the rate of formation of product. An example of such proteins is haloadic dehalogenase- (HAD) like superfamily protein YjG of E. coli (Proudfoot et al., 2004). The calculated values of Kₘ and kₘₐₓ/Kₘ were 0.4 s⁻¹ and 1.1×10⁻⁶ M⁻¹ s⁻¹, respectively.

Figure 4A represents the phylogenetic relationship of CreD and related HD domain phosphohydrolases from other species. Two clusters are evident from the figure. Upper cluster consists of HD domain phosphohydrolases from aerobic, non-motile, mesophilic, Gram-positive bacteria with high G+C content like Rhodococcus wratislaviensis (67.0 mol%), Gordonia rhizosphera (66.8 mol%), and Arthrobacter crystallopoietes (66.6 mol%). CreD belongs to this cluster with phylogenetically very close protein from C. efficiens YS-314 (NPT31166). Lower cluster mostly contains anaerobic, mesophilic, Gram-negative bacteria of low (Emticicia oligotrophica DSM 17448, 36.9 mol%) to high (Loktaneila vestfoidensis, 62.3 mol%) G+C content. Phylogenetic analysis indicates universal existence of CreD homologues.

Figure 1 Metal dependence of phosphohydrolase activity of CreD against 4-NPP (A) before chelation and (B) after chelation with 6.25 μM EDTA (bars represent the standard error of the mean)

Figure 2 Effect of EDTA concentration on phosphohydrolase activity of CreD against 4-NPP (bars represent the standard error of the mean)

Figure 3 Effect of thiol inhibition on phosphohydrolase activity of CreD against 4-NPP (bars represent the standard error of the mean)
Metal-coordinating conserved residues (His\(^{73}\) and Asp\(^{106}\)) and HD signature (His\(^{110}\) and Asp\(^{106}\)) located in CreD sequence are highlighted in figure 4B. Along with Mg\(^{2+}\)-coordinating conserved residue (Asp\(^{106}\)), Zn\(^{2+}\)-chelating residues (His\(^{73}\), His\(^{110}\), Asp\(^{106}\), and Asp\(^{112}\)) are also conserved. However, phosphohydrolase activity towards 4-NPP was negligible with Zn\(^{2+}\) (0.10 µM min\(^{-1}\) mg\(^{-1}\)) as compared to Mg\(^{2+}\) (7.12 µM min\(^{-1}\) mg\(^{-1}\); Figure 1B). In addition, no conserved residues or domains reported to confer signal transduction in phosphohydrolases (Galperin et al., 1999) were located in CreD.

Contribution of HD-domain proteins in nucleic acid metabolism and signal transduction is well-known for more than two decades. This report reveals that CreD of *C. glutamicum*, involved in 4-cresol metabolism, is a metal-dependent phosphohydrolase with conserved HD-domain. To best of our knowledge, involvement of HD-domain phosphohydrolases in aromatic compound degradation has not been reported before.

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REFERENCES


