BIOCHEMICAL AND PHYLOGENETIC STUDIES OF CreD OF *Corynebacterium glutamicum*

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ABSTRACT

CreD characterized as Mg^{2+}-dependent phosphohydrolase with conserved HD domain was involved in 4-cresol metabolism in *Corynebacterium glutamicum*. Native molecular mass of 54 kDa suggested that the biological unit is a dimer. No deoxynucleotide triphosphate triphosphohydrolase (dNTPase) activity was detected for CreD. The apparent K_m and V_{max} values for 4-nitrophenyl phosphate were 0.35 mM and 16.23 µM $min^{-1} mg^{-1}$, respectively, while calculated values for k_{cat} and k_{cat}/K_m were 0.4 s^{-1} and 1.14×10^3 $M^{-1} s^{-1}$, respectively. Among thiol group inhibitors, iodoacetic acid significantly inhibited phosphohydrolase activity. Sequence identity and phylogenetic analysis suggested universal existence of CreD homologues. Involvement of HD-domain hydrolase in aromatic degradation has not been reported before.

Keywords: *Corynebacterium glutamicum*, 4-cresol, HD domain, metal-dependent phosphohydrolase

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-bisphosphate 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 (*ncgl0521* to *ncgl0531*) involved in 4-cresol metabolism has been genetically characterized, which revealed that 4-cresol is metabolized via 4-hydroxybenzoate and protocatechuate 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™ 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-nitrophenyl phosphate (4-NPP) as substrate (**Proudfoot *et al*., 2004**) with divalent cations (Ba^{2+}, Ca^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Ni^{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 10–20 min at 37 °C before recording A_{410}, λ_{max} of 4-nitrophenol product. One enzyme unit was defined as amount of CreD liberating 1 mM of 4-nitrophenol per min. Positive control (with CIP) and negative control (without CreD) were included and all experiments were conducted in triplicate. Non-ion-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 A_{630} (**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 (NCgl0524) 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 (**Aiyar, 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, YfbR of *E. coli* is biologically active as a dimer (**Zimmerman** *et al.***, 2008**), dNTPase of *Enterococcus faecalis* and human PDE4 (**Huai** *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 Ba^{2+}, Ca^{2+} and Mg^{2+} (6.78, 6.45, and 6.78 µM min^{-1} mg^{-1}, respectively) while Cu^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+} 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 Mg^{2+}. Now activity was significantly lower with same concentrations of Ba^{2+}, Ca^{2+}, and Co^{2+}, which was considerably high without chelation (Figure 1A). No considerable activity was observed with Zn^{2+} and in the absence of any cation. Phosphatase activity of SurE and YjjG of *E. coli* with 4-NPP is also Mg^{2+}-dependent (**Proudfoot** *et al.***, 2004**). However, these results are different with those reported for YfbR and tRNA-NT of *E. coli*, and human PDE4 where maximum activity was achieved with Co^{2+}, Ni^{2+}, and Zn^{2+}, 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 (Cys^{47}, Cys^{85}, Cys^{138}, Cys^{159} and Cys^{182}), 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. Iodoacetic acid significantly inhibited phosphohydrolase activity (0.72 µM min^{-1} mg^{-1} at 1 mM concentration) while there was no apparent effect with 10 mM dithiotreitol. 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 K_m and V_{max} as indicated by the plot were 0.35 mM and 16.23 µM min^{-1}mg^{-1}, respectively. The value of K_m placed CreD into the "high K_m" (millimole range) group of phosphohydrolases. Enzyme having high K_m requires a higher concentration of substrate to achieve V_{max}. Under normal conditions, enzyme activity varies with substrate concentration, which consequently controls the rate of formation of product. An example of such proteins is haloacid dehalogenase- (HAD) like superfamily protein YjjG of *E. coli* (**Proudfoot** *et al.***, 2004**). The calculated values of k_{cat} and k_{cat}/K_m were 0.4 s^{-1} and 1.14×10^3 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. effiens* YS-314 (NP737166). Lower cluster mostly contains anaerobic, mesophilic, Gram-negative bacteria of low (*Emticicia oligotrophica* DSM 17448, 36.9 mol%) to high (*Loktanella vestfoldensis*, 62.3 mol%) G+C content. Phylogenetic analysis indicates universal existence of CreD homologues.
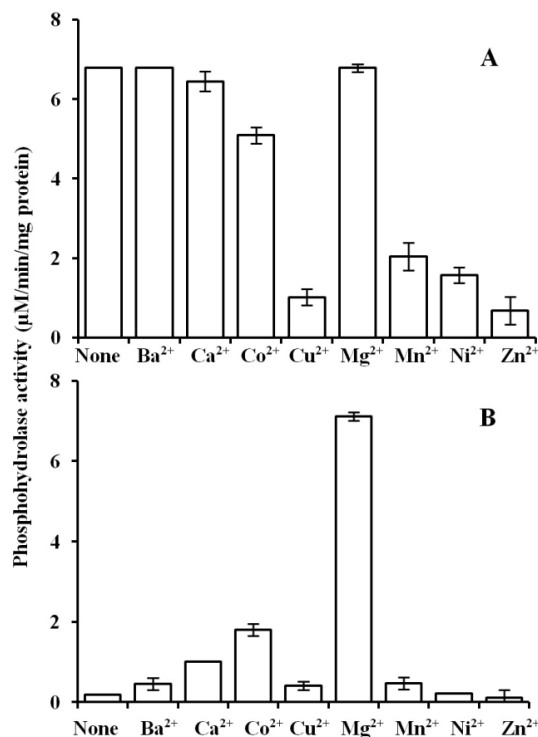

Figure 1 Metal dependence of phosphohydrolase activity of CreD against 4-NPP (A) before chelation and (B) after chelation with 6.25 µM EDTA (*bars* represent the standard error of the mean)
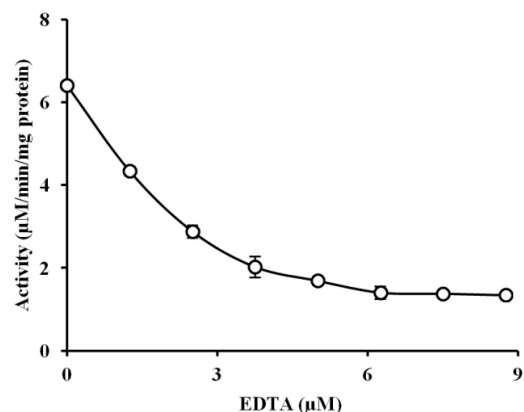

Figure 2 Effect of EDTA concentration on phosphohydrolase activity of CreD against 4-NPP (*bars* represent the standard error of the mean)
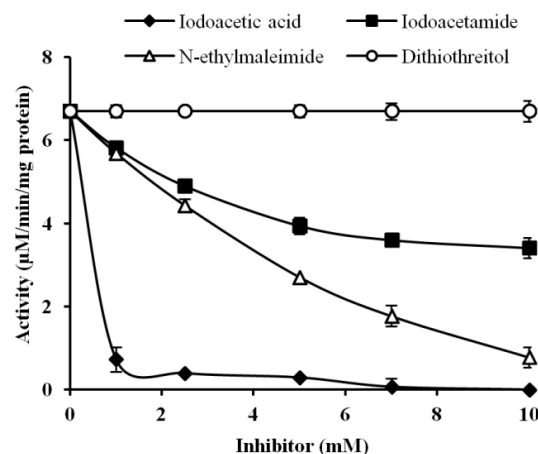

Figure 3 Effect of thiol inhibition on phosphohydrolase activity of CreD against 4-NPP (*bars* represent the standard error of the mean)

Metal-coordinating conserved residues (His[73] and Asp[183]) and HD signature (His[103] and Asp[104]) located in CreD sequence are highlighted in figure 4B. Along with Mg^{2+}-coordinating conserved residue (Asp[104]), Zn^{2+}-chelating residues (His[73], His[103], Asp[104], and Asp[183]) 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.
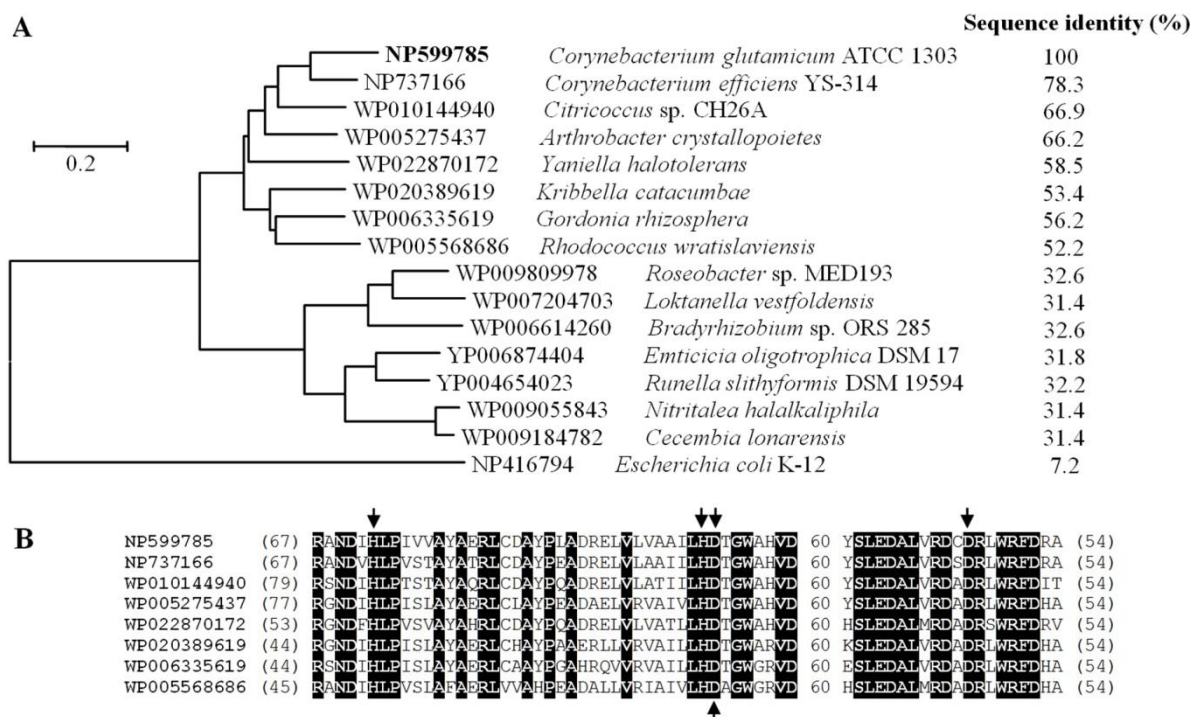

Figure 4 (A) Phylogenetic tree of metal-dependent phosphohydrolases with conserved HD domain including CreD (shown in bold). The scale bar indicates percent divergence (distance). Yfbr of *E. coli* (NP416794) serves as outgroup. (B) Sequence alignment of metal-dependent HD domain phosphohydrolases from Gram-positive, high G+C bacteria. Numbers within parentheses indicate number of residues between protein termini and proximal and distal aligned blocks while numbers between individual blocks indicate number of residues separating them. Highly conserved residues are highlighted in black. Arrows above the alignment show Zn^{2+}-coordinating His and Asp residues while arrow bellow shows Asp residue that binds Mg^{2+}.

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