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ORIGINAL ARTICLE

Chemical rescue of the post-translationally carboxylated lysine mutant of allantoinase and dihydroorotase by metal ions and short-chain carboxylic acids

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Abstract Bacterial allantoinase (ALLase) and dihydroorotase (DHOase) are members of the cyclic amidohydrolase family. ALLase and DHOase possess similar binuclear metal centers in the active site in which two metals are bridged by a post-translationally carboxylated lysine. In this study, we determined the effects of carboxylated lysine and metal binding on the activities of ALLase and DHOase. Although DHOase is a metalloenzyme, purified DHOase showed high activity without additional metal supplementation in a reaction mixture or bacterial culture. However, unlike DHOase, ALLase had no activity unless some specific metal ions were added to the reaction mixture or culture. Substituting the metal binding sites H59, H61, K146, H186, H242, or D315 with alanine completely abolished the activity of ALLase. However, the K146C, K146D and K146E mutants of ALLase were still active with about 1–6 % activity of the wild-type enzyme. These ALLase K146 mutants were found to have 1.4–1.7 mol metal per mole enzyme subunit, which may indicate that they still contained the binuclear metal center in the active site. The activity of the K146A mutant of the ALLase and the K103A mutant of DHOase can be chemically rescued by short-chain carboxylic acids, such as acetic, propionic, and butyric acids, but not by ethanol, propan-1-ol, and imidazole, in the presence of Co^{2+} or Mn^{2+} ions. However, the activity was still $\sim$10-fold less than that of wild-type ALLase. Overall, these results indicated that the 20 natural basic amino acid residues were not sufficiently able to play the role of lysine. Accordingly, we proposed that during evolution, the post-translational modification of carboxylated lysine in the cyclic amidohydrolase family was selected for promoting binuclear metal center self-assembly and increasing the nucleophilicity of the hydroxide at the active site for enzyme catalysis. This kind of chemical rescue combined with site-directed mutagenesis may also be used to identify a binuclear metal center in the active site for other metalloenzymes.

Keywords Allantoinase · Dihydroorotase · Hydantoinase · Imidase · Chemical rescue · Post-translationally carboxylated lysine · Metalloenzyme

Abbreviations

ALLase	Allantoinase
DHOase	Dihydroorotase
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
8-HQSA	8-Hydroxy-5-quinolinesulfonic acid
EDTA	Ethylenediamine tetraacetic acid
IPTG	Isopropyl thiogalactoside
ICP-MS	Inductively coupled plasma mass spectrometry

Introduction

The amidohydrolase superfamily comprises a remarkable set of enzymes that catalyze the hydrolysis of a wide range of substrates bearing amide or ester functional groups at their carbon and phosphorus centers (Gerlt and Babbitt 2001; Seibert and Raushel 2005; Gerlt et al. 2011). The most salient structural landmark for this superfamily of

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hydrolytic enzymes is a mononuclear or binuclear metal center embedded within the confines of a $(\beta/\alpha)_8$-barrel structural fold (Seibert and Raushel 2005). Based on their functional and structural similarities to related enzymes, hydantoinase (HYDase), allantoinase (ALLase), dihydropyrimidinase (DHPase), and dihydroorotase (DHOase) are deemed to belong to the cyclic amidohydrolase family (Holm and Sander 1997; Kim and Kim 1998). In spite of possessing functional similarity, their mutual amino acid identities appear to be relatively low. These metal-dependent enzymes catalyze the hydrolysis of the cyclic amide bond of each substrate in either five- or six-membered rings in the metabolism of purines and pyrimidines (Fig. 1).

ALLase exists in a wide variety of organisms, including bacteria, fungi, and plants, as well as a few animals. ALLase catalyzes the reversible hydrolysis of allantoin to allantoic acid, which is a key reaction in the biosynthesis and degradation of ureide required for the utilization of nitrogen in purine-derived compounds (Hayashi et al. 2000). Two forms of ALLase exist: one is metal independent and initially annotated as a polysaccharide deacetylase (Ramazzina et al. 2008); the other is a homotetrameric dinuclear metalloenzyme (Kim et al. 2000, 2009). Recombinant metal-dependent ALLase isolated from a culture without added metal contains a significant amount of iron but possesses very low activity (Mulrooney and Hausinger 2003). Fe-ALLase can be significantly activated when incubating with Co^{2+} or Mn^{2+} ions (Ho et al. 2011), suggesting that the Fe^{2+} ion(s) in the active sites can be replaced. In addition, the chelator inhibition of ALLase by 8-hydroxy-5-quinolinesulfonic acid (8-HQSA), but not by EDTA, is pH dependent (Ho et al. 2011).

DHOase catalyzes the reversible cyclization of carbamoyl aspartate to dihydroorotate in the third step of the de novo biosynthesis pathway of pyrimidine nucleotides

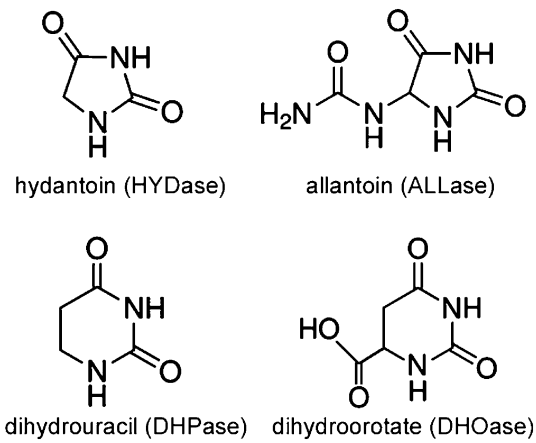

Fig. 1 Substrate of the cyclic amidohydrolase family: hydantoinase (HYDase), allantoinase (ALLase), dihydropyrimidinase (DHPase), and dihydroorotase (DHOase)

(Evans and Guy 2004). In mammals, this enzyme has one metal per monomer (Huang et al. 1999) and is found as part of the large multifunctional protein carbamoyl phosphate synthetase/aspartate transcarbamoylase/dihydroorotase (CAD) (Christopherson and Jones 1980; Mally et al. 1980; Evans and Guy 2004; Nara et al. 2012). However, in prokaryotic organisms, CADs are usually expressed separately and function independently (Washabaugh and Collins 1984) or form multifunctional complexes (McPhail and Shepherdson 2006; Zhang et al. 2009; Nara et al. 2012). DHOase from bacteria also contains 2–3 metals (Brown and Collins 1991), two of which are located in the active site (Thoden et al. 2001). The native Zn^{2+} ions in the active site of DHOase can be substituted with Co^{2+} or Cd^{2+} ions by reconstitution of the apoenzyme with divalent cations in the presence of bicarbonate (Porter et al. 2004). Unlike ALLase (Mulrooney and Hausinger 2003; Kim et al. 2009; Ho et al. 2011) and HYDase (Huang et al. 2009), DHOase is still active even without any additional metal supplementation in growth medium or assay mixture (Wang et al. 2010).

The active site of ALLase (Kim et al. 2009) and DHOase (Thoden et al. 2001) revealed by crystal structures contains four histidine and one aspartate residues that are required for metal binding and catalytic activity. Many, but not all, cyclic amidohydrolases (including ALLase and DHOase) also have two metals bridged by a post-carboxylated lysine at their active sites (Thoden et al. 2001; Abendroth et al. 2002; Cheon et al. 2002; Xu et al. 2003; Martin et al. 2005; Radha Kishan et al. 2005; Lohkamp et al. 2006; Kim et al. 2009; Martinez-Rodriguez et al. 2010a; Mehboob et al. 2010). For example, DHOase from *Bacillus anthracis* uses aspartate instead (Mehboob et al. 2010). The carboxylation on lysine is a chemical modification involving carbon dioxide in solution (Park and Hausinger 1995). The presence of carboxylated lysine in HYDase is found to play a role in binuclear metal center self-assembly (Huang et al. 2009) and increases the nucleophilicity of the hydroxide for catalysis (Kumar et al. 2011). Nevertheless, the absolute necessity of lysine in the cyclic amidohydrolase family is unclear. HYDase is an industrial enzyme used to produce some important intermediates for the preparation of semi-synthetic antibiotics (Altenbuchner et al. 2001; Martinez-Rodriguez et al. 2010b). Although HYDase, ALLase, and DHOase have a similar metallocenter, their substrate specificities do not overlapped and the metal effects on these enzymes differ. The effects of post-translational lysine carboxylation on the activity and metal binding of ALLase and DHOase are still unknown. This information is essential for formulating a general catalytic mechanism for the cyclic amidohydrolase family. In this study, we determined the effects of carboxylated lysine and metal binding on the activity of

ALLase and DHOase. The kind of chemical rescue combined with site-directed mutagenesis presented in this study may also be used to identify a binuclear metal center in the active site of other metalloenzymes.

Materials and methods

Materials

All restriction enzymes and DNA-modifying enzymes were purchased from New England Biolabs (Ipswich, MA, USA), unless otherwise stated. All custom oligonucleotide primers were obtained from Invitrogen Corporation (Carlsbad, CA, USA). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

Construction of ALLase and DHOase expression plasmids

STM0523, the gene encoding the putative ALLase, was PCR amplified using the genomic DNA of *Salmonella enterica* serovar Typhimurium LT2 (McClelland et al. 2001) as the template. The forward (5′-CGCGTCATATGTCTTTTGATTTAATTA-3′) and reverse (5′-TTTAACTCGAGCTGCTGATGTTTAAGG-3′) primers were designed to introduce unique NdeI and XhoI restriction sites (underlined) into ALLase, permitting the insertion of the amplified gene into the pET21b vector (Novagen Inc., Madison, WI, USA) for the protein expression in *E. coli* BL21. The construction of pET21e-*Kp*DHO expression vector for *Klebsiella pneumoniae* DHOase expression has been previously described (Wang et al. 2010).

Site-directed mutagenesis

The ALLase mutants were generated using a QuikChange Site-Directed Mutagenesis kit according to the manufacturer's protocol (Stratagene; LaJolla, CA, USA). The construction of the DHOase K103A mutant has been previously described (Wang et al. 2010). The presence of the mutation was verified by DNA sequencing in each construct. The oligonucleotide primers for the preparation of mutants were: 5′-ATGGTTGACGCGGCTACCCACATTTCT-3′ and 5′-AGAAATGTGGGTAGCCGCGTCAACCAT-3′ for ALLase H59A; 5′-TTGACGCGCATACCGCCATTTCTGAAC-3′ and 5′-GTTCAGAAATGGCGGTATGCGCGTCAA-3′ for ALLase H61A; 5′-TTGTCGGCTTCGCGTGCTTTGTCGCCA-3′ and 5′-TGGCGACAAAGCACGCGAAGCCGACAA-3′ for ALLase K146A; 5′-GTTGTCGGCTTCTGTTGCTTTGTCGCC-3′ and 5′-CAACAGCCGAAGACAACGAAACAGCGG-3′ for ALLase K146C; 5′-GTTGTCGGCTTCGATTGCTTTGTCGCC-3′ and 5′-CAACAGCCGAAGCTAACGAAACAGCGG-3′ for ALLase K146D; 5′-GTTGTCGGCTTCGAGTGCTTTGTCGCC-3′ and 5′-GGCGACAAAGCACTCGAAGCCGACAAC-3′ for ALLase K146E; 5′-GTTGTCGGCTTCTACTGCTTTGTCGCC-3′ and 5′-GGCGACAAAGCAGTAGAAGCCGACAAC-3′ for ALLase K146H; 5′-ACGGTACTGGTAGCTTGCGAAAATGCT-3′ and 5′-AGCATTTTCGCAAGCTACCAGTACCGT-3′ for ALLase H186A; 5′-AGGTTGCCGTGTCGTATCAGCAGCCCG-3′ and 5′-CGGGCTGCTGATACGACACGGCAACCA-3′ for ALLase H242A; 5′-TGCCTGGTATCCGCCCACTCTCCATGC-3′ and 5′-GCATGGAGAGTGGGCGGATACCAGGCA-3′ for ALLase D315A; 5′-TGTTCACCGCCGCCGCACTCTACCCGG-3′ and 5′-CCGGGTAGAGTGCGGCGGCGGTGAACA-3′ for DHOase K103A. The underlined sequences denote the mutated amino acid.

Protein expression and purification

Recombinant proteins were expressed and purified using a previously described protocol (Huang et al. 2006, 2009, 2012; Hsieh and Huang 2011; Huang and Huang 2012). In a typical procedure, *Escherichia coli* BL21(DE3) cells were individually transformed with the plasmid by the heat shock method, and then grown to 0.9 OD_{600} at 37 °C in Luria-Bertani medium containing 250 μg/mL ampicillin (and with or without 1 mM metal ions as indicated) with rapid shaking. Overexpression of the construct was induced by incubating with 1 mM isopropyl thiogalactoside (IPTG) for 3 h at 37 °C. The cells overexpressing the protein were chilled on ice, harvested by centrifugation, resuspended in buffer A (20 mM Tris–HCl, 5 mM imidazole, and 0.5 M NaCl, pH 7.9) and disrupted by sonication with ice cooling. The protein purified from the soluble supernatant by Ni^{2+}-affinity chromatography (HiTrap HP; GE Healthcare Bio-Sciences, Piscataway, NJ, USA) was eluted with buffer B (20 mM Tris–HCl, 250 mM imidazole, and 0.5 M NaCl, pH 7.9) and dialyzed against a dialysis buffer (50 mM HEPES and 50 mM NaCl, pH 7.0). Protein purity remained greater than 95 % as determined by Coomassie-stained SDS-PAGE (Mini-PROTEAN Tetra System; Bio-Rad, CA, USA).

Protein concentration

The protein concentrations of enzyme solution were determined by the bicinchoninic acid protein assay using bovine serum albumin as a standard (Smith et al. 1985).

Enzyme assay

A rapid spectrophotometric assay was used to determine the enzymatic activity according to a previously described

protocol for ALLase (Ho et al. 2011), DHOase (Wang et al. 2010), imidase (Huang and Yang 2002, 2003), and HYDase (Huang et al. 2009). In a typical procedure, the hydrolysis of allantoin and dihydroorotate was measured at 25 °C as the decrease in absorbance at 258 and 230 nm, respectively. To start the reaction, purified protein (5 mg/mL) was preincubated with 1 mM metal ions at room temperature for 4 min, and then the protein solution (1–30 μg) was added to a 2 mL solution containing the substrate (40 mM allantoin or 1 mM dihydroorotate) and 100 mM Tris–HCl (pH 8.0). The extinction coefficients of allantoin and dihydroorotic acids were 0.0261 $mM^{-1}{\cdot}cm^{-1}$ at 258 nm and 0.92 $mM^{-1}{\cdot}cm^{-1}$ at 230 nm, respectively. Substrate hydrolysis was monitored with a UV/vis spectrophotometer (Hitachi U 3300, Hitachi High-Technologies, Tokyo, Japan). The initial rates of change were a function of enzyme concentration within the absorbance range of 0.01–0.3 min^{-1}. A unit of activity was defined as the amount of enzyme catalyzing the hydrolysis of 1 μmol substrate/min, and the specific activity was expressed in terms of units of activity per milligram of enzyme.

Chemical rescue experiment

The restoration activities of the K146A, K146C, K146D, K146E, and K146H mutants of the ALLase and the DHOase K103A mutant were determined in the presence of short-chain carboxylic acids and Co^{2+} or Mn^{2+} ions. The reaction mixture included the mutant protein, metal ions (1 mM), various concentrations of the carboxylic acid, and reagents needed for the standard assay. The control experiments included various concentrations of carboxylic acids in the absence of metal ions. Carboxylic acids were dissolved in the assay buffer with the pH adjusted.

Metal content

Inductively coupled plasma-mass spectrometry (ICP-MS) (Elan 5000, PerkinElmer, USA) was used to measure the concentration of each cation in the protein samples. Prior to ICP-MS analysis, the protein solution was passed through a HiTrap desalting column (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) and eluted with HEPES buffer (10 mM, pH 7.0) at 4 °C. After elution, almost all DHOases immediately precipitated within several minutes, whereas ALLase still remained soluble and stable for at least a week. The measurements for each sample were repeated five times and the standard deviation was calculated. Normally, two or more samples were used for each determination by ICP-MS. Less than 1 mL of enzyme sample (0.05–0.2 mg/mL) was used for each determination.

Results and discussion

Purification of ALLase and DHOase

Salmonella enterica serovar Typhimurium LT2 ALLase and *Klebsiella pneumoniae* DHOase were hetero-overexpressed in *E. coli* and purified from the soluble supernatant by Ni^{2+}-affinity chromatography (Fig. 2). Pure protein was obtained in this single chromatographic step with an elution of buffer B (20 mM Tris–HCl, 250 mM imidazole, and 0.5 M NaCl, pH 7.9) in high yield (~68 % for ALLase and ~61 % for DHOase). Approximately >80 mg of purified protein was obtained from 1 L of a culture of *E. coli* cells in both cases. The mutant proteins were also purified according to the same protocol as that for the wild-type proteins, and with very similar purification results.

Metal-activated ALLase

S. typhimurium ALLase was cloned, expressed, and purified, but the catalytic activity of the purified ALLase was undetected. Some metal ions were added to the reaction mixture. Table 1 shows that the addition of 1 mM $MnCl_2$, $CoCl_2$, or $ZnCl_2$ activated the ALLase activity and followed the order $Mn^{2+} > Co^{2+} > Zn^{2+}$; $CdCl_2$, $NiCl_2$, $MgCl_2$, and $CaCl_2$ were not useful.

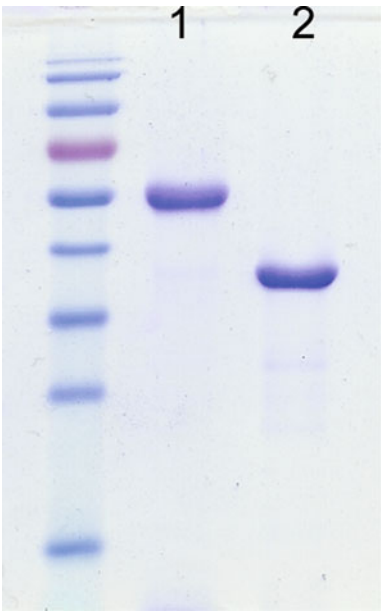

Fig. 2 Coomassie Blue-stained SDS-PAGE (12 %) of purified *Salmonella enterica* serovar Typhimurium LT2 ALLase (*lane 1*), *Klebsiella pneumoniae* DHOase (*lane 2*), and molecular mass standards. The sizes of the standard proteins, from the *top down*, are as follows: 170, 130, 100, 70, 55, 40, 35, 25, and 20 kDa. The purified ALLase migrated near the 55 kDa standards, and DHOase migrated between the 35 and 40 kDa standards on the SDS-PAGE

Metal-amended ALLase

We also added some metal ions into the bacterial culture for ALLase expression, and every resultant metal-amended ALLase was purified and analyzed. We found that supplementation with $CoCl_2$, $MnCl_2$, $ZnCl_2$, $CdCl_2$ or $NiCl_2$ activated the ALLase activity and followed the order $Co^{2+} > Mn^{2+} > Zn^{2+} > Cd^{2+} > Ni^{2+}$ (Table 1). This case significantly differed from those of the metal-activated enzymes; $CdCl_2$ and $NiCl_2$ were useful and $CoCl_2$ was the best supplement. Co^{2+}-amended ALLase was also the most active among the metal-amended and -activated ALLases.

Metal-(de)activated DHOase

Although DHOase is a metalloenzyme, the purified DHOase showed high activity without additional metal supplementation in the reaction mixture or culture (Table 2). This result differed from that of ALLase (Ho et al. 2011) and HYDase (Huang et al. 2009). ALLase and HYDase exhibited no activity without additional metal supplementation. The addition of metal ions (1 mM) to the reaction mixture was found to inhibit DHOase activity. The inhibitory effect of metal ions on the DHOase activity followed the order Cd^{2+}, $Zn^{2+} > Co^{2+} > Mn^{2+} > Ni^{2+}$ ions; Mg^{2+} and Ca^{2+} had no effect.

Metal-amended DHOase

Although DHOase was active without additional metal supplementation, some specific metal ions added to the culture were found to increase the DHOase activity substantially. Table 2 shows that every metal-amended DHOase was purified and analyzed, whereas $MnCl_2$, $CoCl_2$, $NiCl_2$, and $MgCl_2$ supplements were found to have a positive effect on the DHOase activity. The activity of DHOase purified from the culture containing $ZnCl_2$ or $CdCl_2$ (1 mM) was lower than that without adding metal. Taken together, Co^{2+}-amended DHOase was the most active among the metal-amended and -(de)activated DHOases (Table 2).

Metal contents of the metal-amended ALLase

The metal contents of the metal-amended ALLase are shown in Table 3. Mixtures of metals were found in purified metal-amended ALLase, even without any metal supplement. Significant amounts of Fe (1.0 ± 0.2 mol of metal per mole of enzyme subunit) were observed in recombinant ALLase obtained from metal-unsupplemented cell culture. The addition of $MnCl_2$, $CoCl_2$, and $ZnCl_2$ in the cell culture significantly affected the metal content of ALLase. Generally, the total metal content of the purified metal-amended ALLases ranged from around 1.6 (Zn^{2+}-amended enzyme) to 2.0 (Co^{2+}-amended enzyme) metal per subunit. Although excess metal ions were added to the cell culture, these resultant ALLases still contained high amounts of Fe. Fe in these metal-amended ALLases may come from the LB medium. Interestingly, Zn^{2+} is

Table 1 The specific activity of the metal-activated and metal-amended ALLases

Metal ions	Specific activity[a] (μmol/mg/min)	
	Metal-activated ALLase[b] (as included in the assay)	Metal-amended ALLase[c] (as included in the culture)
Without adding metal	$<10^{-3}$	$<10^{-3}$
$MnCl_2$	61 ± 4	47 ± 3
$CoCl_2$	6.0 ± 1.8	74 ± 5
$ZnCl_2$	1 ± 0.3	17 ± 3
$CdCl_2$	$<10^{-3}$	8 ± 2
$NiCl_2$	$<10^{-3}$	2 ± 0.3
$MgCl_2$	$<10^{-3}$	$<10^{-3}$
$CaCl_2$	$<10^{-3}$	$<10^{-3}$

[a] 40 mM allantoin was used for the specific activity of the ALLase. The errors are standard deviation determined at 3–4 measurements

[b] Purified ALLase was preincubated with the indicated metal ions (1 mM) for 4 min, and then analyzed by the standard assay

[c] The bacterial culture for ALLase expression was supplemented with the indicated metal ions (1 mM). ALLase was then purified and analyzed by the standard assay

Table 2 The specific activity of the metal-activated and metal-amended DHOases

Metal ions	Specific activity[a] (μmol/mg/min)	
	Metal-(de) activated DHOase[b] (as included in the assay)	Metal-amended DHOase[c] (as included in the culture)
Without adding metal	7.2 ± 0.4	7.2 ± 0.4
$MnCl_2$	3.0 ± 0.3	10.3 ± 0.8
$CoCl_2$	1.4 ± 0.1	24.1 ± 1.1
$ZnCl_2$	1.3 ± 0.1	5.5 ± 0.7
$CdCl_2$	1.3 ± 0.1	2.1 ± 0.3
$NiCl_2$	7.0 ± 0.2	15.1 ± 1.6
$MgCl_2$	7.2 ± 0.3	20.2 ± 1.0
$CaCl_2$	7.2 ± 0.3	7.0 ± 0.7

[a] 1 mM dihydroorotate was used for the specific activity of the DHOase. The errors are standard deviation determined at 3–4 measurements

[b] Purified DHOase was preincubated with the indicated metal ions (1 mM) for 4 min, and then analyzed by the standard assay

[c] The bacterial culture for DHOase expression was supplemented with the indicated metal ions (1 mM). DHOase was then purified and analyzed by the standard assay

Table 3 Metal contents of the metal-amended ALLase

Metal added in cell culture	Metal per enzyme subunit				
	Zn	Co	Mn	Fe	Total
Unsupplemented	0.2 ± 0.1	0.0 ± 0.0	0.1 ± 0.1	1.0 ± 0.2	1.3
$MnCl_2$	0.1 ± 0.1	0.0 ± 0.0	1.6 ± 0.2	0.2 ± 0.1	1.9
$CoCl_2$	0.2 ± 0.1	1.2 ± 0.3	0.1 ± 0.1	0.5 ± 0.2	2.0
$ZnCl_2$	1.2 ± 0.2	0.0 ± 0.0	0.2 ± 0.1	0.2 ± 0.2	1.6

The metal contents in ALLase were determined by inductively coupled plasma mass spectrometry (ICP-MS). The enzymes were purified from metal-amended cultures. Prior to ICP-MS analysis, the protein solution was passed through a HiTrap desalting column and eluted with HEPES buffer (10 mM, pH 7.0) at 4 °C

presumably the native metal ion in ALLase (Mulrooney and Hausinger 2003), and not the major metal found in recombinant ALLase from unsupplemented medium or the metal that gave the highest enzymatic activity (Table 1). Despite the lower bioavailability of Co and Mn than Zn and Fe, the in vivo biological role of Co and Mn in ALLase should not be completely ruled out at this time.

In this study, we only analyzed the activity of the metal-amended DHOases but did not determine the number of specific metals bound to DHOase such as those in ALLase because of its low stability. The purified metal-amended DHOases immediately precipitated probably because of oxidation (Washabaugh and Collins 1986). After eluting in a desalting column for further metal content analysis, almost all DHOases precipitated within a few minutes. Our laboratory is currently screening some redox agents to study the stability of DHOase.

Metal binding sites of ALLase and DHOase

ALLase and DHOase are close members of the amidohydrolase family (Kim and Kim 1998; Thoden et al. 2001; Kim et al. 2009). The metal binding properties of ALLase and DHOase investigated in this study considerably differed (Tables 1, 2). However, the crystal structural information indicated similar active sites for metal binding: H59, H61, a carboxylated K146, H186, H242, and D315 for ALLase; and H17, H19, a carboxylated K103, H140, H178, and D251 for DHOase (Fig. 3a). To assess the contribution of these residues, the alanine substitution was constructed and analyzed. As expected, activities of the H59A, H61A, K146A, H186A, H242A, and D315A mutants of ALLase were severely impaired (Table 4). These positions for metal binding in 65 sequenced ALLases from different organisms were perfectly conserved (Fig. 3b). The contribution of these structurally corresponding residues of DHOase has also been previously determined (Wang et al. 2010). Thus, the binuclear metal center is essential to the ALLase and DHOase activities.

However, the usual post-translationally carboxylated lysine is not always conserved in the structures of the cyclic amidohydrolase family, such as the cases of HYDase from *Bacillus* sp. AR9 (Radha Kishan et al. 2005) as well as DHOases from *Aquifex aeolicus* (Martin et al. 2005) and *B. anthracis* (Mehboob et al. 2010). To determine the role of the post-translationally carboxylated lysine in the active site of ALLase, we constructed and analyzed several K146 mutants, K146C, K146D, K146H, and K146E; each mutant had a possible ligand for metal binding. Table 4 shows that the K146 mutants of ALLase (K146C, K146D, and K146E), believed to be unable to possess a long carboxylated side chain as lysine, were still active but had only about 1–6 % activity of wild-type ALLase. The activity of the K146H mutant of ALLase was also totally undetected possibly because histidine was able to coordinate only with a single metal ion.

Metal content of the Co^{2+}-amended ALLase K146 mutants

Although cystine, aspartate, glutamate, and histidine were potential metal binding ligands, the ALLase K146C, K146D, and K146E mutants had significantly low activities (Table 4). The K146A and K146H mutants were inactive. To determine whether the binuclear metal center in the active site of the K146A, K146C, K146D, K146E, and K146H mutants still self-assembled in the same manner as that in wild-type ALLase (their native carboxylated sites were eliminated), we detected the metal contents in these mutants by ICP-MS. Table 5 shows that the Co^{2+}-amended ALLase K146A contained 0.3 metal per subunit, and the other K146 mutants were found to have 1.0–1.7 metal per subunit, all less than that of wild-type ALLase ($\sim$2.0 metal per subunit). These data indicated the formation of a binuclear metal center in the active site of the K146C, K146D, and K146E mutants, but not in K146A and K146H. However, K146C, K146D, and K146E still exhibited very low activities compared with wild-type ALLase. Adding 1 mM $CoCl_2$ to the assayed reaction did

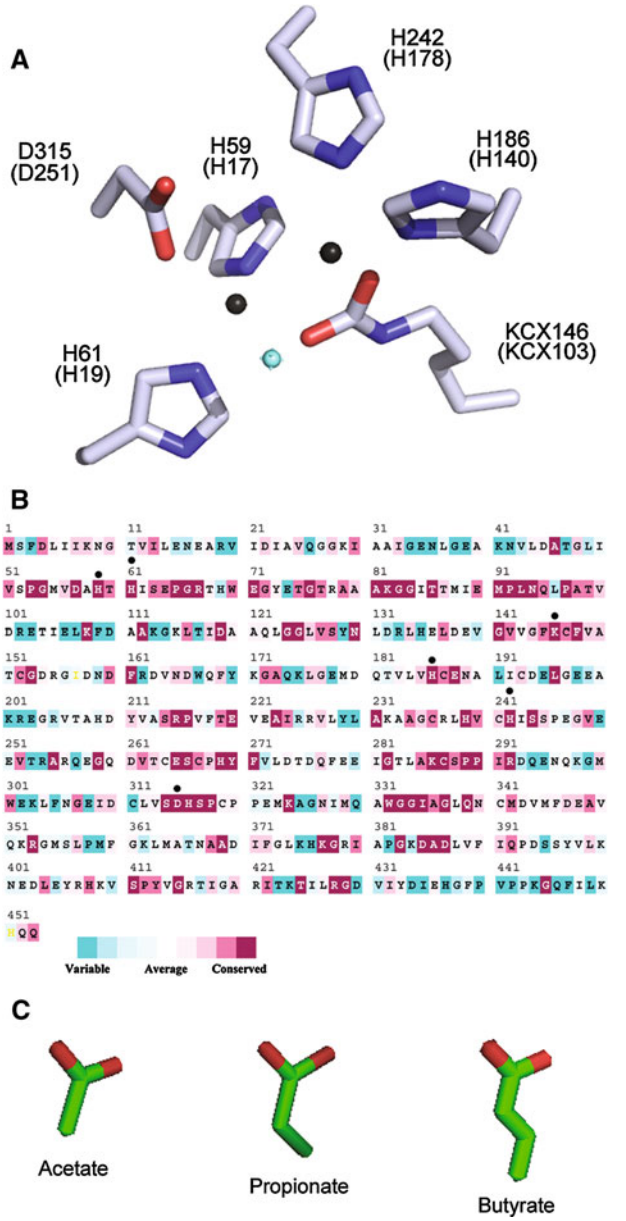

Fig. 3 **a** The binuclear metal center within the active site of ALLase and DHOase (in *parentheses*). The coordinate was obtained from the Protein Data Bank (entry 3E74). KCX, a post-carboxylated lysine. The metal ions (in *black*) and a metal-bound water molecule (in *light blue*) are also shown. **b** An alignment consensus of 65 sequenced ALLase homologs by ConSurf (Landau et al. 2005) reveals the degree of variability at each position along the primary sequence. Amino acid residues that are highly variable are colored teal, while highly conserved amino acid residues are colored burgundy. A consensus sequence was established by determining which amino acid residue is most commonly found at each position relative to the primary sequence of *S. typhimurium* ALLase. Residues of the consensus sequence marked with a *black dot* are metal binding ligands. Note that these positions are perfectly conserved. **c** Structure of carboxylic acids, acetate, propionate, and butyrate. These carboxylic acids were used for chemical rescue of the post-translationally carboxylated lysine mutant of ALLase and DHOase (color figure online)

Table 4 Mutational analysis and chemical rescue of purified ALLase mutants

	Specific activity[a] (μmol/mg/min)	Relative activity (%)
Co^{2+}-wt (culture)[b]	74 ± 5	100
Co^{2+}-wt (assay)[c]	6.0 ± 1.8	8.1
wt[b,c]	$<10^{-3}$	0
Co^{2+}-H59A[b,c]	$<10^{-3}$	0
Co^{2+}-H61A[b,c]	$<10^{-3}$	0
Co^{2+}-K146A[b,c]	$<10^{-3}$	0
K146A-acetic acid[b,c]	$<10^{-3}$	0
K146A-propionic acid[b,c]	$<10^{-3}$	0
K146A-butyric acid[b,c]	$<10^{-3}$	0
Co^{2+}-K146A-acetic acid[c,d]	2.5 ± 0.5	3.4
Co^{2+}-K146A-propionic acid[c,d]	7.2 ± 0.9	9.7
Co^{2+}-K146A-butyric acid[c,d]	1.7 ± 0.3	2.3
Co^{2+}-K146C[b]	4.5 ± 0.3	6.1
Co^{2+}-K146C-propionic acid[c,e]	4.3 ± 0.4	5.8
Co^{2+}-K146D[b]	2.9 ± 0.4	3.9
Co^{2+}-K146D-propionic acid[c,e]	3.1 ± 0.2	4.2
Co^{2+}-K146E[b]	0.7 ± 0.2	0.9
Co^{2+}-K146E-propionic acid[c,e]	0.6 ± 0.1	0.8
Co^{2+}-K146H[b,c]	$<10^{-3}$	0
Co^{2+}-K146H-propionic acid[c,e]	$<10^{-3}$	0
Co^{2+}-H186A[b,c]	$<10^{-3}$	0
Co^{2+}-H242A[b,c]	$<10^{-3}$	0
Co^{2+}-D315A[b,c]	$<10^{-3}$	0

[a] 40 mM allantoin was used for the specific activity of the ALLase. The errors are standard deviation determined at 3–4 measurements

[b] The bacterial culture for ALLase expression was supplemented with $CoCl_2$ (1 mM). ALLase was then purified and analyzed by the standard assay

[c] Purified ALLase was preincubated with the indicated metal ions (1 mM) for 4 min, and then analyzed by the standard assay

[d] The maximal activity is shown

[e] 10 mM propionic acid and 1 mM $CoCl_2$ were used for chemical rescue. Note that the resultant activity for these mutants almost did not significantly change compared with that assayed in the absence of propionic acid and Co^{2+} ions

not affect/increase the activity of the Co^{2+}-amended mutants (K146A, K146C, K146D, K146E, and K146H). Aspartate and glutamate both have a carboxylic acid side chain, and K146D and K146E may have a (partial) binuclear metal center at the active site (Table 5). However, only carboxylated lysine was highly preferred for the catalytic activity of ALLase. Therefore, we concluded that other amino acid residues cannot replace lysine in ALLase and it was perfectly conserved during evolution (Fig. 3b).

The metal center in the amidohydrolase superfamily is vital for enzymatic activity (Seibert and Raushel 2005).

Table 5 Metal contents of the Co^{2+}-amended ALLase K146 mutants

K146 mutant	Metal per enzyme subunit				
	Zn	Co	Mn	Fe	Total
K146A	0.0 ± 0.0	0.2 ± 0.1	0.0 ± 0.0	0.1 ± 0.1	0.3
K146C	0.3 ± 0.1	0.8 ± 0.0	0.1 ± 0.1	0.2 ± 0.1	1.4
K146D	0.1 ± 0.1	1.0 ± 0.0	0.1 ± 0.1	0.2 ± 0.1	1.4
K146E	0.2 ± 0.1	1.1 ± 0.3	0.1 ± 0.1	0.3 ± 0.1	1.7
K146H	0.1 ± 0.0	0.8 ± 0.1	0.0 ± 0.0	0.1 ± 0.1	1.0

The metal contents in these ALLase mutants were determined by inductively coupled plasma mass spectrometry (ICP-MS). The mutant enzymes were purified from Co^{2+}-amended cultures. Prior to ICP-MS analysis, the protein solution was passed through a HiTrap desalting column and eluted with HEPES buffer (10 mM, pH 7.0) at 4 °C

A mononuclear or binuclear metal center is the structural landmark for hydrolytic enzymes with the triosephosphate isomerase (TIM)-barrel structural fold (Gerlt and Raushel 2003). Mutation for the metal-binding ligand may disrupt the correct binuclear metal center formation and/or cause a slightly change in the catalytic orientation in the active site of ALLase. Mutation of the metal binding ligand within the binuclear metal center of HYDase is also found to be partially eliminated in a metal binding site and produces inactive enzyme (Huang et al. 2009). Some of these lysine mutants of HYDase and ALLase may remain as the binuclear metal center, which was insufficient for catalysis unlike carboxylated lysine-directed assembly.

Chemical rescue of K146A mutant of ALLase by short-chain carboxylic acids

To characterize further the requirement of the carboxylated lysine in the active site of ALLase, we reconstituted the K146A mutant of ALLase using metal ions and a carboxylic acid, such as acetic, propionic, or butyric acids (Fig. 3c), into the reaction mixture. Indeed, the activity of the K146A mutant of ALLase can be dramatically reactivated by adding the carboxylic acid and Co^{2+} (Fig. 4a) or Mn^{2+} ions (Fig. 4b); their maximal activities were about 2–10 % of the wild-type ALLase activity. However, after desalting, the activity of the chemically rescued K146A mutant was undetected. Thus, these carboxylic acids may facilitate assembly of the binuclear metal center only temporarily. The hydrolysis rate of allantoin was proportional to the enzyme concentration in this rescuing experiment. In addition, when the K146A mutant was omitted from this assay, no activity was observed. A high concentration of these short-chain carboxylic acids (~30 mM) was needed to obtain the maximal activity of the Co^{2+}-K146A mutant of ALLase. The concentrations of the carboxylic acids needed to yield the maximum activity of the Mn^{2+}-K146A mutant of ALLase were 5, 20, and 10 mM acetic, propionic, and butyric acids, respectively. Too high concentration of carboxylic acids (more than 50 mM) caused irreversible precipitation of the K146A mutant of ALLase. Notably, the activity of the rescuing Co^{2+}-K146A mutant of ALLase was not correlated with the length of carboxylic acid and followed the order propionic acid > acetic acid > butyric acid.

Chemical rescue of K103A mutant of DHOase by short-chain carboxylic acids

The activity of the K103A mutant of DHOase has been previously known to be completely abolished (Wang et al. 2010). In this study, we attempted to restore its activity

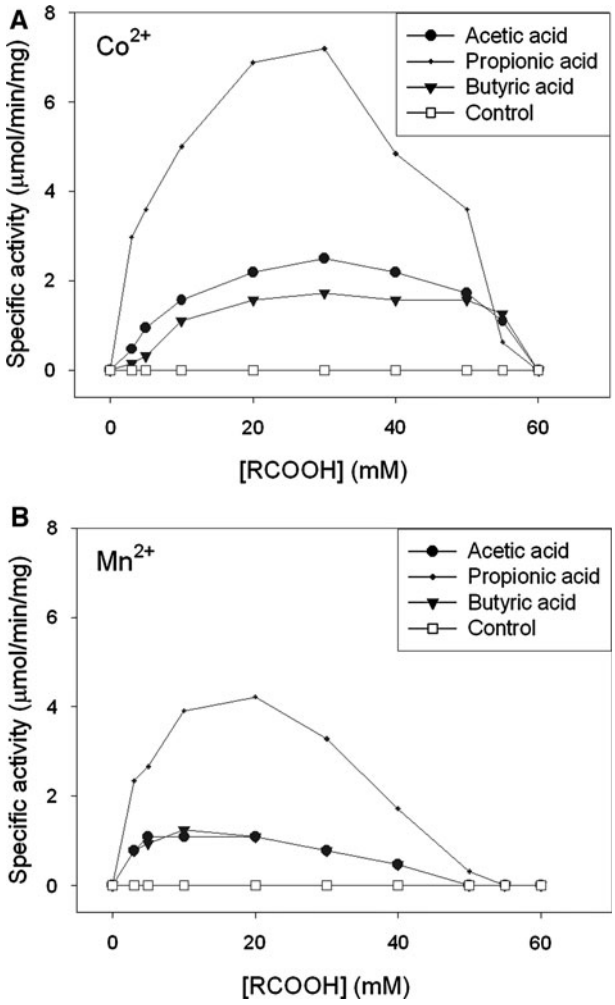

Fig. 4 Chemical rescue of the K146A mutant of ALLase by short-chain carboxylic acids and **a** Co^{2+} or **b** Mn^{2+} ions. The reaction mixture included the K146A, the metal ions (1 mM), various concentrations of the carboxylic acid, and reagents needed for the standard assay. The control experiments included various concentrations of the carboxylic acids in the absence of the metal ions. Carboxylic acids were dissolved in the assay buffer with the pH adjusted

similar to the case of the ALLase K146A mutant. As shown in Fig. 5, the activity of K103A mutant of DHOase can be restored by adding carboxylic acid and Co^{2+} (Fig. 5a) or Mn^{2+} ions (Fig. 5b), and the results were similar to those of ALLase K146A. We also used ethanol, propan-1-ol, or imidazole for chemical rescue such as the carboxylic acid in both cases of the Co^{2+}-K146A mutant of ALLase and the Co^{2+}-K103A mutant of DHOase, but no significant effect on activity was observed.

Chemical rescue of K146C, K146D, K146E, and K146H mutants of ALLase by short-chain carboxylic acids

To determine whether the activity of the other ALLase K146 mutants, namely, K146C, K146D, K146E, and K146H, can be enhanced by short-chain carboxylic acids and metal ions, their chemical rescues were also examined with 10 mM propionic acid and 1 mM $CoCl_2$ (Table 4) as that for K146A. However, unlike the case of K146A, the resultant activity for these mutants almost did not significantly change compared with that assayed in the absence of propionic acid and Co^{2+} ions. Overall, given that K146A contained a low level of metal (Table 5), we believe that the K146A mutant did not form the binuclear metal center and thus the alanine side chain may leave sufficient space for short-chain carboxylic acids and metal ions to enter the active site; entering short-chain carboxylic acids may fill the position occupied by the missing carboxylated lysine to build an artificial binuclear metal center for catalysis. These data also agreed with the analysis results for the metal content of these K146C, K146D, K146E, and K146H mutants. They contained more than one metal per enzyme subunit (Table 5), suggesting the formation of a full or partial binuclear metal center. Thus, the chemical rescue did not produce any effect on these K146C, K146D, K146E, and K146H mutants.

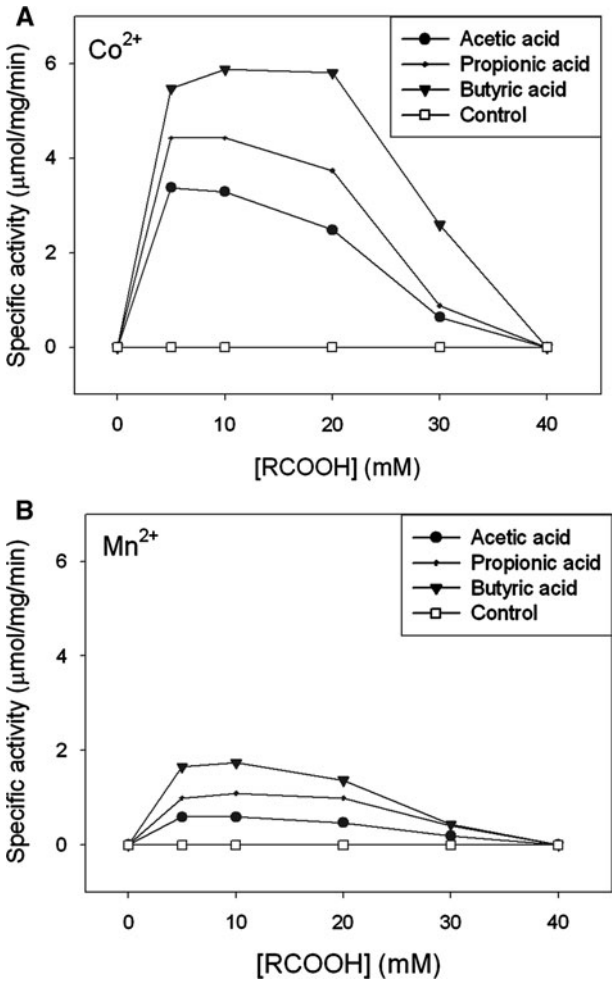

Fig. 5 Chemical rescue of the K103A mutant of DHOase by short-chain carboxylic acids and **a** Co^{2+} or **b** Mn^{2+} ions. The reaction mixture included the K103A, the metal ions (1 mM), various concentrations of the carboxylic acid, and reagents needed for the standard assay. The control experiments included various concentrations of the carboxylic acids in the absence of the metal ions. Carboxylic acids were dissolved in the assay buffer with the pH adjusted

Post-carboxylated lysine was required for increased activity of the cyclic amidohydrolase family

In this study, we found that the length of the metal binding ligand was not an important factor affecting higher rescuing activity of the lysine mutant of ALLase (Fig. 4). The maximal activity of the reconstituted K146A enzyme was also still ~-folds less than that of wild-type ALLase. One of two possible reasons was that short-chain carboxylic acids can only partially rescue the enzymes because they cannot completely occupy the active center as did carboxylated lysine. Although the concentrations of short-chain carboxylic acids used in this study were >1000-folds higher than those of ALLase mutants, the short-chain carboxylic acids were not necessary to be docked in the active site or bind with ALLase with high affinity. Thus, we were unsure whether every enzyme contained the carboxylic acid and metal ions after chemical rescue during the assay. The other reason may be the different chemical properties between carbamate and carboxylate (Fig. 6). A resonance structure of carbamate results in both oxygen atoms forming negative charges (Fig. 6a), perhaps promoting di-metal assembly. This phenomenon may also increase the nucleophilicity of the hydroxide in the active site for catalysis. A partially positive charge was unavailable for carboxylate (Fig. 6b). Consequently, the K146 mutants of ALLase, K146C (Fig. 6c), K146D (Fig. 6b), and K146E (Fig. 6b) were still active, although their activities were significantly decreased compared with that of wild-type ALLase (Table 4). Thus, the post-carboxylated

lysine was required for increased activity of the cyclic amidohydrolase family, and this requirement was unchanged during evolution.

Post-carboxylated lysine was selected as a novel amino acid for enzyme catalysis

This study showed that the absence of the post-carboxylated lysine remarkably affected the catalytic activities of ALLase and DHOase, similar to the activity of phosphotriesterase (Kuo et al. 1997), urease (Pearson et al. 1998), and HYDase (Huang et al. 2009), which are members of the amidohydrolase superfamily. This study further indicated that the post-carboxylated lysine in ALLase replaced by some other potential metal ligands may still contain a self-assembled di-metal center (Table 5). Apart from the amidohydrolase superfamily, the same post-modification on lysine was also found in the β-lactamase family but with different phenomena. Unlike ALLase and other amidohydrolases, the post-carboxylated lysine in OXA-1 and -10 β-lactamases were used to form a hydrogen bond with the serine nucleophile (Li et al. 2005; Schneider et al. 2009), but not to promote di-metal binding. The carboxylated lysine in these β-lactamases mutated to aspartate and glutamate led to a nearly inactive enzyme, suggesting a unique role of lysine in these enzyme families.

In conclusion, studies on mutation and successful chemical rescues for the K146A mutant of ALLase and the K103A mutant of DHOase, as well as similar results for β-lactamases, indicated that the 20 natural basic amino acid residues may not be negatively charged sufficiently as carboxylated lysine to aid these kinds of enzyme catalysis. During evolution, the post-translational modification of carboxylated lysine may thus be selected as a novel amino acid for increased activity of the cyclic amidohydrolase and β-lactamase families. We still cannot rule out the possibility that the post-translational carboxylation on lysine in ALLase and DHOase may be a limiting step for the regulation of the entire metabolic pathway of purines and pyrimidines.

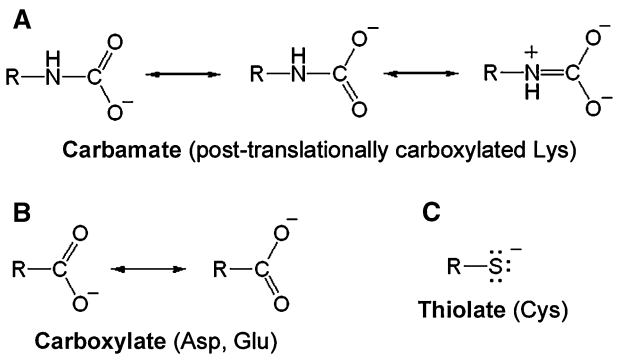

Fig. 6 **a** Resonance forms for carbamate of carboxylated Lys. **b** Resonance forms for carboxylate, the side chain of Asp and Glu. **c** Thiolate ion, the side chain of deprotonated Cys

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Conflict of interest None declared.

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