

Catalytic Zinc Site and Mechanism of the Metalloenzyme PR-AMP Cyclohydrolase

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Supporting Information

ABSTRACT: The enzyme *N*¹-(5'-phosphoribosyl) adenosine-5'-monophosphate cyclohydrolase (PR-AMP cyclohydrolase) is a Zn²⁺ metalloprotein encoded by the *hisI* gene. It catalyzes the third step of histidine biosynthesis, an uncommon ring-opening of a purine heterocycle for use in primary metabolism. A three-dimensional structure of the enzyme from *Methanobacterium thermoautotrophicum* has revealed that three conserved cysteine residues occur at the dimer interface and likely form the catalytic site. To investigate the functions of these cysteines in the enzyme from *Methanococcus vannielii*, a series of biochemical studies were pursued to test the basic hypothesis regarding their roles in catalysis. Inactivation of the enzyme activity by methyl methane thiosulfonate (MMTS) or 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) also compromised the Zn²⁺ binding properties of the protein inducing loss of up to 90% of the metal. Overall reaction stoichiometry and the potassium cyanide (KCN) induced cleavage of the protein suggested that all three cysteines were modified in the process. The enzyme was protected from DTNB-induced inactivation by inclusion of the substrate *N*¹-(5'-phosphoribosyl)adenosine 5'-monophosphate; (PR-AMP), while Mg²⁺, a metal required for catalytic activity, enhanced the rate of inactivation. Site-directed mutations of the conserved C93, C109, C116 and the double mutant C109/C116 were prepared and analyzed for catalytic activity, Zn²⁺ content, and reactivity with DTNB. Substitution of alanine for each of the conserved cysteines showed no measurable catalytic activity, and only the C116A was still capable of binding Zn²⁺. Reactions of DTNB with the C109A/C116A double mutant showed that C93 is completely modified within 0.5 s. A model consistent with these data involves a DTNB-induced mixed disulfide linkage between C93 and C109 or C116, followed by ejection of the active site Zn²⁺ and provides further evidence that the Zn²⁺ coordination site involves the three conserved cysteine residues. The C93 reactivity is modulated by the presence of the Zn²⁺ and Mg²⁺ and substantiates the role of this residue as a metal ligand. In addition, Mg²⁺ ligand binding site(s) indicated by the structural analysis were probed by site-directed mutagenesis of three key aspartate residues flanking the conserved C93 which were shown to have a functional impact on catalysis, cysteine activation, and metal (zinc) binding capacity. The unique amino acid sequence, the dynamic properties of the cysteine ligands involved in Zn²⁺ coordination, and the requirement for a second metal (Mg²⁺) are discussed in the context of their roles in catalysis. The results are consistent with a Zn²⁺-mediated activation of H₂O mechanism involving histidine as a general base that has features similar to but distinct from those of previously characterized purine and pyrimidine deaminases.



Histidine is the only amino acid that derives carbon and nitrogen from another advanced metabolite in the form of ATP. As part of this process, *N*¹-(5'-phosphoribosyl) adenosine-5'-monophosphate cyclohydrolase (PR-AMP cyclohydrolase) encoded by the *hisI* gene maintains the unique position of promoting the ring-opening event of a purine heterocycle for use in primary metabolism and constitutes the third step of histidine biosynthesis (Scheme 1). This metabolically intense process is dependent upon the utilization of carbon and nitrogen through this hydrolytic event. The potential significance for inhibitor design of this pathway at the level of the PR-AMP cyclohydrolase has been proposed.^{1–3} Our efforts have focused on a monofunctional enzyme from *Methanococcus vannielii* and established that this enzyme is a metalloprotein containing one high affinity Zn²⁺ per subunit and requiring a free Mg²⁺ ion for catalytic activity.⁴ There are analogous enzymes belonging to the class of zinc hydrolases

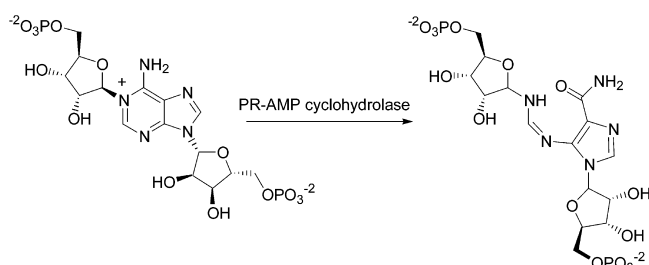
that catalyze purine or pyrimidine deamination, for example, adenosine,^{5,6} cytidine,⁷ blastocidin S,⁸ and cytosine deaminases.⁹ The three-dimensional structures of these enzymes have revealed the details of the zinc coordination.^{10–17} These enzymes provide specific metal ligands to promote activation of water molecules in the sphere of the zinc atom for attack of the substrate heterocycle.¹⁸ However, there are some differences between these deaminases and PR-AMP cyclohydrolase, the foremost being the regiochemistry of the bond cleavage event. PR-AMP cyclohydrolase displays a pH rate profile for k_{cat}/K_m that implicates a single enzyme species with a $\text{p}K_a$ of 7.3.⁴ This feature is in contrast with those for either cytidine deaminase

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Scheme 1. Conversion of PR-AMP to 5'-ProFAR by PR-AMP Cyclohydrolase



(pK_a 5.4)⁷ or adenosine deaminase (pK_a 4.7).¹⁹ Finally, there is no amino acid sequence homology between the known cyclohydrolases and deaminases implicating distinct evolutionary processes but some elements in common at the level of catalytic mechanism.

Our initial characterization of PR-AMP-cyclohydrolase established that the functional loss of catalytic activity was correlated with an irreversible loss of Zn^{2+} .⁴ While no evidence for direct involvement of the Zn^{2+} binding site in catalysis was revealed in these previous studies, the functional similarity to the deaminases implicates a role for the metal binding sites in catalysis. Analysis of the sequence conservation in the PR-AMP cyclohydrolases from various organisms provided evidence for potential Zn^{2+} protein ligands. Global alignments of the HisI amino acid sequences by us and others²⁰ indicate that three cysteine and aspartate residues are invariant in all species and occur in a stretch of highly conserved amino acid residues (see pfam01502.12). The functional importance for the conserved cysteine residues within this region follow from the sequence analysis of the known *Escherichia coli* UTH903 *hisI*(-) mutant.²¹ DNA sequencing of this mutant indicated that the *hisIE* gene had undergone a point mutation that rendered a C to F change at the third conserved cysteine residue.

Despite the apparent significance of these conserved cysteine residues in PR-AMP cyclohydrolase, no functional analyses of these residues have been reported. The discovery of the metalloprotein character of the enzyme from *M. vannielii*, and the recent three-dimensional structure of the *Methanobacterium thermoautotrophicum* protein indicating cysteine residues from separate monomers making the Zn^{2+} binding site motivated a working hypothesis for evolution of the catalytic site at the dimer interface.^{22,23} All three of the conserved cysteines are implicated in coordination of the metal in a Cd^{2+} substituted form of the protein. An additional metal binding site for a Mg^{2+} ion was also proposed based upon the known requirement for catalytic activity and the proximity to the putative Zn^{2+} binding site.

The distinctive features of the active site in the PR-AMP cyclohydrolase implicated role(s) for the conserved cysteines in catalysis. By using chemical modification and analysis of site-directed mutants of the invariant cysteine and histidine residues as well as some other key aspartate residues in the *M. vannielii* PR-AMP cyclohydrolase, the reactivity and essential features of the amino acids in metal binding were evaluated. The unique amino acid sequence and the dynamic properties of the cysteine Zn^{2+} ligands are discussed in the context of their likely direct role(s) in catalysis. We also establish experimental evidence for the functional roles of three aspartate residues in metal binding and propose a catalytic mechanism consistent with a distinct zinc hydrolase family.

■ MATERIALS AND METHODS

Materials. All chemicals, buffers, resins, and general enzymes were purchased from commercial sources. Custom oligonucleotides were synthesized by the Purdue Campus facility. DNA preparation was performed using commercial kits. Desalting and concentration of proteins were completed using commercial centrifugal filters.

General Methods. All procedures, restriction digests, transformation of *E. coli*, end-labeling, and general molecular biology manipulations were carried out using standard protocols.^{24,25} *E. coli* DH5 α or XL-1 Blue were used as host strains for propagation of plasmid DNA. Manual DNA sequencing of the mutant constructs was performed using a commercial cycle sequencing kit with end-labeled T7 primer. After separation of DNA sequencing reaction fragments on an 8% PAGE urea gel, the results were imaged on a phosphorimager. Complete DNA sequencing of the mutant *hisI* genes was performed using fluorescent 5'Cy5 end-labeled primers (standard T7 or T3 primers) and cycle sequencing.

Protein concentrations were determined by the method of Bradford.²⁶ SDS-PAGE was run under reducing conditions using Tris-Tricine gels as described previously.⁴ UV-vis spectrophotometry, stopped-flow spectrophotometry, and MALDI-MS were performed in house. Atomic absorption analyses of protein samples for Zn^{2+} content were conducted at the Indiana State Chemist Laboratory.

Amino Acid Sequence Alignments. All amino acid sequences presented here were taken from the available databases (DNA and protein) at the National Center for Biotechnology Information (NCBI). Homologous sequences were identified using BLAST sequence search algorithm.^{27,28} Sequence alignments provided were produced using ClustalW (v1.6)²⁹ after initial alignment by Pile-Up.^{30,31}

Mutant Preparation. The C93A, C109A, C116A, and H110A mutants were produced using the megaprimer PCR³² method, while the C109A/C116A, D92E, D94E, and D94A mutants were produced using the sight overlap extension method.³³ The C109A/C116A double mutant was produced in succession utilizing the C116A mutant as template for the C109A change. When possible these changes were chosen to incorporate or remove unique cleavage sites to allow for initial screening by DNA restriction analysis.

The native primers, which contained restriction sites in noncoding regions, were as follows: *M. vannielii hisI* (Sense) 5'-GCA TGA ATT CAT ATG GGC ATT AAA GAC ATT-3', (Antisense) 5'-GGG CAA GCT TCA TTA ATC TAG TTT ATC TCC-3'. These primers were designed with *EcoRI* and *NcoI* sites and a start codon in the sense primer and a *HindIII* site and a stop codon in the antisense primer. Sense strand mutant primers, where the underlined codon indicates the position of the mutation, were used for C93A 5'-GAA GTA TTT AAA GAC GCG GAT GGT GAT GCC CTC-3', C116A 5'-GGA TAT ATG TCA GCT TTT CAT AAT AAA-3', H110A 5'-GCA TGG GCA TGT GCA GAA GGA TAT ATG-3'; and D94A 5'-GTA TTT AAA GAC TGT GCA GGT GAT GCC CTC-3'. In a similar fashion, antisense primers were used for C109A 5'-ATA TCC TTC ATG AGC TGC CCA TCC AGT-3'. D92E 5'-ATC ACC ATC ACA TTC TTT AAA TAC TTC-3'; and D94E 5'-GAG GGC ATC ACC TTC ACA CTG TTT AAA-3'.

All final products were cloned into pBluescript II SK(+) using the *EcoRI* and *HindIII* restriction sites, and selected

recombinants were analyzed by DNA restriction and sequence analysis. The site directed mutations were confirmed using a [^{32}P]-end labeled primer and the Cycle Sequencing kit (Promega). The entire coding sequences of the *hisI* mutant clones were then established using the general DNA sequencing methods. The *NcoI*–*HindIII* restriction fragments encoding each mutant *hisI* were ligated into the same sites of pET-28(b)+ to create the desired *hisI* mutant expression constructs.

Overproduction of Protein, Purification, and Assay of PR-AMP Cyclohydrolase. Overproduction, purification, and assay of PR-AMP cyclohydrolase were carried out using the procedure previously described.⁴ Since many of the mutants possessed no catalytic activity, SDS–PAGE was used to follow the elution of the mutant proteins at each step of the purification. These samples were concentrated, filtered through a 0.22 μm low protein-binding filter, and stored at 4 °C until use.

PR-AMP Cyclohydrolase Inactivation by Cysteine Modification. Fresh solutions of methyl methane thiosulfonate (MMTS) and recrystallized 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) were used for inactivation studies with PR-AMP cyclohydrolase. MMTS inactivation reactions contained 2.0 μM enzyme in 50 mM Tris-HCl pH 7.5, 0.5 mM EDTA, and varied concentrations of MMTS. When substrate, PR-AMP was present in reactions it was at 1.1 mM. The experiments were initiated by the addition of MMTS to the protein sample at 4 °C. Samples (10 μL) were taken at specified time intervals, immediately diluted into 1 mL of assay buffer, and analyzed using the standard enzyme assay conditions. Pseudo first-order rates of inactivation were estimated from the percent activity as a function of time. All initial rates were estimated in duplicate and parallel controls were run for all samples.

The inactivation of PR-AMP cyclohydrolase by DTNB was executed in 50 mM Tris-HCl pH 7.5, 1 mM EDTA, 10 mM Mg^{2+} at 20 °C. To a sample of PR-AMP cyclohydrolase (2.0 μM) at 20 °C was added DTNB (106 μM to 296 μM), and when appropriate 1.1 mM PR-AMP was included. At indicated times, 10 μL aliquots of these mixtures were removed and immediately diluted into 1 mL assay buffer and analyzed for catalytic activity. Estimates of the inactivation rates were derived as described above.

To compare the rates of nitro-5-thiobenzoate anion (TNB^{2-}) formation with those for enzyme inactivation, TNB^{2-} formation was monitored in separate experiments using the conditions as described above. DTNB was mixed with PR-AMP cyclohydrolase (9.0 μM) to final concentrations of 585 μM , 981 μM , or 1.36 mM, and formation of TNB^{2-} was observed at 412 nm for the full course of the reaction. The data were fit to either single or double exponential functions using Origin 5.0 software to arrive at estimates of the rate constants.

Samples of the MMTS and DTNB inactivated PR-AMP cyclohydrolase were submitted for metal analysis (see below). The samples were allowed to react for up to 1 h at which point less than 0.01% the catalytic activity remained.

Stoichiometry of DTNB Modification. The stoichiometry of modification by DTNB for native and mutant PR-AMP cyclohydrolases was determined by incubation of varied enzyme concentrations (9.7 to 23.4 μM) with 30–100-fold molar excess DTNB, in 50 mM Tris-HCl pH 8.5, 1 mM EDTA, 5–10 mM Mg^{2+} at 30 °C. All PR-AMP cyclohydrolase preparations used in these determinations were buffer exchanged by gel filtration chromatography using Superose

12 or Sephadex-G25 columns before analysis. The reactions required from 15 to 20 min for completion. Fresh solutions of recrystallized DTNB were used for all stoichiometry experiments and a determination of the extinction coefficient for TNB^{2-} under these conditions yielded 13830 $\text{M}^{-1}\text{cm}^{-1}$.³⁴

The DTNB modified PR-AMP cyclohydrolase was subjected to gel filtration over Superose 12 or Sephadex-G25 to remove excess DTNB. These samples were then reacted with KCN (5–10 mM) in the same buffer as above to release TNB^{2-} . Release of TNB^{2-} was rapid under these conditions and end point absorbances at 412 nm were used to estimate the amount released. Samples of cyanylated-PRAMP cyclohydrolase produced from KCN treatment (or by direct reaction with NTCB) were cleaved at the sites of modification and analyzed by MALDI-MS analysis.^{35,36} Briefly, to samples of the cyanylated-PR-AMP cyclohydrolase was added Tris-base (3 M), to adjust the pH to 9.0 and the reaction was allowed to react at 37 °C for 16 h to affect cleavage. Control reactions were untreated protein under the same conditions. The reactions were then diluted 10–50 times with 50% ACN/0.1% TFA (1–10 pmol μL^{-1} protein) and submitted for MALDI-MS to determine the sites of modifications.

Stopped-Flow Kinetics. The initial rate estimates of TNB^{2-} formation for reactions of DTNB with native PR-AMP cyclohydrolase, the C109A/C116A double mutant species, and free cysteine were determined using a Hi-Tech stopped-flow spectrophotometer. These experiments were executed using a single mixing mode with a stop volume of 110 μL at 20 ± 0.1 °C. Final buffer conditions were 50 mM Tris-HCl pH 8.5, 0.5 mM EDTA 1 mM MgCl_2 , or 50 mM KH_2PO_4 pH 7.3, 0.5 mM EDTA and 1 mM MgCl_2 . After mixing, the reactions contained 440 μM DTNB and 8.7 μM of enzyme or cysteine. The reactions were observed at 412 nm and the resultant data (triplicate) were averaged and fit using a sequential first-order rate model in KinetAsyst 2.0 (Hi-Tech, Inc.) to arrive at estimates for the rate constants.

Stopped-Flow Observation of the PR-AMP Cyclohydrolase Reaction. The UV profile for the PR-AMP cyclohydrolase enzymatic reaction was monitored from 220 to 340 nm at 23 °C using a Hi-Tech stopped-flow spectrophotometer in single-mixing mode. Syringes contained solutions of PR-AMP (4 μM) with Mg^{2+} (5 mM) and PR-AMP cyclohydrolase (4 μM) in 50 mM Tris-HCl pH 7.5, 1 mM EDTA. Spectra for the enzyme, product, and substrate starting solutions were acquired. Multivariate analysis of the resultant data was performed using Specfit Software Ver. 2.1.^{37,38}

Metal Ion Analysis. All samples of PR-AMP cyclohydrolase were prepared by extensive dialysis at 4 °C for 2 days prior to metal ion analyses by ICP emission spectroscopy and atomic absorption spectroscopy (AAS). The dialysis bags contained up to 10 mg of protein at 1.0 mg mL^{-1} and equilibrated in 400 mL of 30 mM HEPES pH 7.5 containing 4 g L^{-1} Chelex 100 (50–100 mesh, Na^+ form) resin for 6 to 8 h, followed by changes after 12–16 h (2 L) and 30–34 h (2 L). Spectra/Por 4 12000–14000 MWCO dialysis membranes was prepared according to standard procedures³⁹ and rinsed with HEPES buffer prepared by stirring with 4 g L^{-1} Chelex 100 for 24 h before use. All HEPES solutions were made from the sodium salt and titrated with NaOH to pH 7.5. All containers (plastic and glassware) were triple rinsed, or soaked in HEPES containing Chelex 100 (4 g L^{-1}), and plastic materials were used whenever possible. As a control to correct for any matrix effects a standard curve was

the three identical positions C93, C109, and C116 are also the only cysteine residues in the protein. The importance of C116 was indicated by the finding that in the *E. coli* UTH903 *hisI*(-) mutant the *hisIE* gene contains the C116F mutation that rendered the HisIE protein inactive.²¹ Together with sequence conservation, these data implicated key functional roles for the three invariant residues at C93, C109, and C116 in the cyclohydrolase motif and provided further impetus for a focused study.

In addition to the three cysteines, three aspartates and a histidine are also conserved and identical within the PR-AMP cyclohydrolase sequences. Using a composite profile derived from this alignment, [LIV]-x(S)-D-C-D-x-D-x-L-x(7)-[GN]-x(2,3)-C-H-x(4)-[ST]-C-F, the Prosite database⁴² was searched for additional homologous sequences, especially those related to known catalytic or metal binding sites; however no homologous sequences were found. More focused comparisons were based upon the possible mechanistic relationships with cysteine proteases and to the reaction catalyzed by adenosine deaminase. Alignments with different forms of adenosine deaminase and AMP deaminase showed no significant similarities with respect to active site residues. The PR-AMP cyclohydrolase sequence contains all residues required for a cysteine protease; a number of papain-like cysteine proteases were aligned using a partial active site signature sequence.^{43,44} Sequence alignments based upon the metalloproteases active site pentapeptide motif H-E-X-X-H⁴⁵ were also investigated. The results from these efforts failed to reveal any significant relationships between PR-AMP cyclohydrolase and functionally related metalloproteins or cysteine proteases or with any of the described most common active site zinc ligand arrangements.^{18,46-49}

Recently, the X-ray crystal structure of the Cd²⁺-substituted enzyme from *M. thermoautotrophicum* was solved and revealed that these residues occur at the dimer interface.²² All of the cysteines and aspartates implicated from the sequence alignments also appear coordinated with Cd²⁺, one metal coordinates with three cysteines and a second metal with the three aspartates. However, the lack of detectable sequence homology with other zinc hydrolases indicated the cyclohydrolase motif as unique and further motivated detailed biochemical analyses concentrating on the role of the conserved cysteines and the second metal binding site.

Inactivation of PR-AMP Cyclohydrolase by Cysteine Specific Reagents. Our initial focus on the conserved cysteines was to determine how they play a role in the activity of PR-AMP cyclohydrolase. To this end, the inactivation of PR-AMP cyclohydrolase was assayed at varying concentrations of MMTS with and without substrate, PR-AMP. As shown in Figure 2, this reagent in the absence or presence of exogenous Mg²⁺ caused a time-dependent inactivation of PR-AMP cyclohydrolase. However, inclusion of the substrate PR-AMP at 1.1 mM protected the enzyme from inactivation 14-fold over enzyme alone at identical concentrations of MMTS. In contrast, N¹-[(5'-phosphoribulosyl) formimino]-5-aminocarboxamide ribonucleotide adenosine monophosphate (5'-ProFAR) (the product) at similar concentrations had only a modest 2-fold impact on the rate of the MMTS inactivation reactions (data not shown).

The thiol modification reagent DTNB was also used to analyze the reactive cysteines in PR-AMP cyclohydrolase (Figure 3). The presence of the substrate PR-AMP also afforded protection to the enzyme reducing the rate 5-fold at

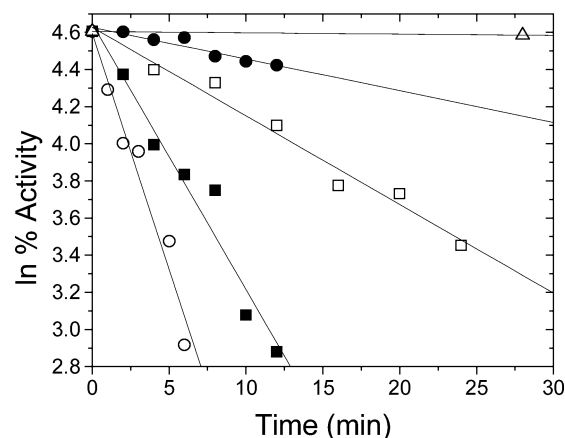


Figure 2. MMTS inactivation of PR-AMP cyclohydrolase and effect of substrate on reactivity of cysteines. Data are as follows (Δ) not determined, control no MMTS; (●) $1.7 \times 10^{-2} \text{ min}^{-1}$, 78 μM MMTS, 1.1 mM PR-AMP; (□) $4.8 \times 10^{-2} \text{ min}^{-1}$, 39 μM MMTS; (■) $1.5 \times 10^{-1} \text{ min}^{-1}$, 59 μM MMTS; (○) $2.6 \times 10^{-1} \text{ min}^{-1}$, 78 μM MMTS.

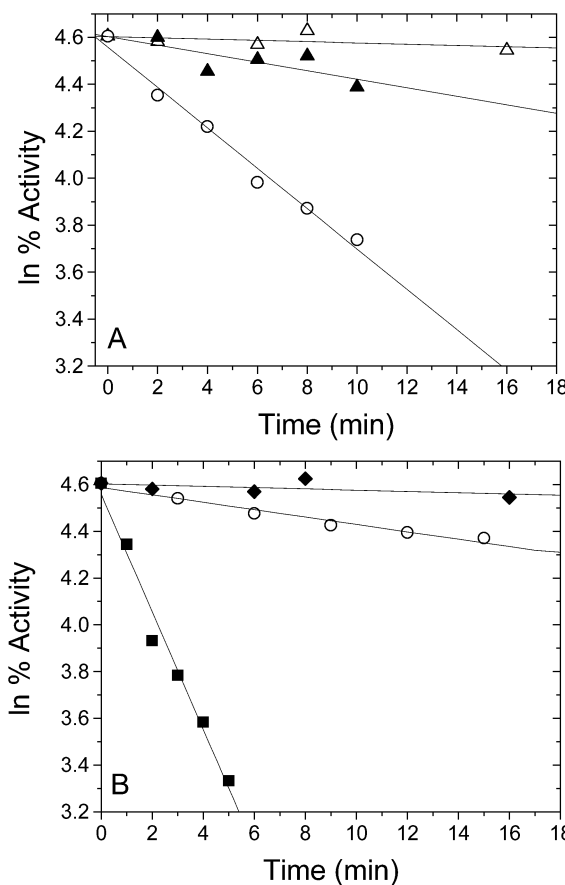


Figure 3. DTNB inactivation rates of PR-AMP cyclohydrolase and the effect of PR-AMP and Mg²⁺. Data are as follows (A) (Δ) $2.7 \times 10^{-3} \text{ min}^{-1}$, control no DTNB, (○) $8.5 \times 10^{-2} \text{ min}^{-1}$, 212 μM DTNB, (▲) $1.82 \times 10^{-2} \text{ min}^{-1}$, 212 μM DTNB with 1.0 mM PR-AMP; (B) (◆) $2.7 \times 10^{-3} \text{ min}^{-1}$, control no DTNB, (○) $1.6 \times 10^{-2} \text{ min}^{-1}$, in the absence of Mg²⁺, 424 μM DTNB, (■) $2.5 \times 10^{-1} \text{ min}^{-1}$, 424 μM DTNB.

identical concentrations of DTNB (Figure 3A). Our previous studies established that Mg²⁺ was required for catalytic activity. In contrast to the MMTS reaction, the rate of the DTNB

inactivation was also dependent upon Mg^{2+} shown as a 16-fold enhancement in Figure 3B.

DTNB Reaction Stoichiometry and Sites of Modification with PR-AMP Cyclohydrolase. PR-AMP cyclohydrolase was reacted with DTNB to ascertain the stoichiometry of accessible cysteine residues (Table 1). Samples of the

Table 1. Stoichiometry of Reaction with DTNB

native cyclohydrolase (specific activity) U/mg	stoichiometry (TNB ²⁻ /subunit)	stoichiometry (KCN release/subunit)
10.2	1.12 ± 0.01	0.52 ± 0.01
13.1	1.60 ± 0.10	0.65 ± 0.03
18.1	1.98 ± 0.15	0.72 ± 0.05.

cyclohydrolase with high specific activity (18 U mg⁻¹) resulted in a stoichiometry of 2.0 ± 0.2 mol of TNB²⁻ per mole of subunit. The DTNB modified PR-AMP cyclohydrolase was passed through a gel filtration column and was treated with KCN to release the TNB²⁻ from the modified cysteine residues. However, the mole ratio of TNB²⁻ released was never greater than one cysteine per subunit. To analyze the positions of the DTNB modification, several independent samples were further processed to affect cleavage at the site of cyanylation.³⁵ MALDI-MS analysis of these fragment mixtures provided evidence that all three conserved cysteines in PR-AMP cyclohydrolase could undergo cyanylation; the relative abundance of each site was not determined. The results indicate that DTNB modification can occur at all the sites but with differing frequency. However, incorporation of a third equivalent of DTNB was not observed.

Catalytic Activity, Metal Content, and DTNB Reactions of PR-AMP Cyclohydrolase Cysteine Mutants. Subsequently, we focused on understanding the role of the three conserved cysteines in metal binding and catalysis in PR-AMP cyclohydrolase. The original observations regarding the pH dependence of the steady rate constants implicated a functional group with a pK_a of 7.3 that was required for catalytic activity.⁴ This functional pK_a was not affected by substitution of the enzyme with Cd²⁺.²² The chemical identity of this functional group could be a metal-associated water molecule and/or protein metal ligands. In order to directly assess the essential features of the three invariant cysteines, DNA coding sequences in the *M. vannielii hisI* were created that changed these residues to alanine (C93A, C109A, C116A). In addition, based upon the initial results a double alanine mutant C109A/C116A was also created for this analysis. Each of these mutant proteins were expressed in *E. coli* and purified. In all cases, these proteins showed identical behavior during the purification steps but showed no detectable activity. The location of protein containing fractions was verified by the use of SDS-PAGE electrophoresis. The purified mutants were each tested at levels up to 1000-fold higher than those used for the native enzyme in the standard initial velocity assays. Using the calculated lower limit of detection for this assay, it could be deduced that the cysteine mutant proteins had catalytic activities that were reduced by >10⁴-fold when compared to the native enzyme.

The Zn²⁺ contents of the DTNB modified as well as mutant forms of PR-AMP cyclohydrolase were analyzed using ICP-ES or atomic absorption spectroscopy (AAS). Since the cysteine modifying reagents DTNB and MMTS were shown to modify all three conserved cysteine residues, the Zn²⁺ content of the modified wild type PR-AMP cyclohydrolase was analyzed. The

results in Table 2 indicate that the cysteine-modified protein had a measurable loss of Zn²⁺ content. To assess the individual

Table 2. Zinc Content and Kinetics of PR-AMP Cyclohydrolase Preparations

enzyme species	Zn ²⁺ (mol/subunit)	k _{cat} /K _m (M ⁻¹ s ⁻¹)
PR-AMP cyclohydrolase	1.1, 0.88, 1.07	4.1 ± 0.8 × 10 ⁵
D92E	0.72, 0.51	2.3 ± 0.2 × 10 ³
C93A	0.003, 0.020	inactive
D94A	0.60, 0.43	inactive
D94E	0.65, 0.63	1.8 ± 0.2 × 10 ²
C109A	0.37, 0.22	inactive
H110A	1.16, 1.09	8.3 ± 2 × 10 ²
C116A	0.99, 0.90	inactive
C109/116A	0.027, 0.007	inactive
DTNB or MMTS ^a	0.27, or 0.19 ^a , 0.52 ^a	

^aThe average percent deviation for the independent Zn²⁺ measurements is 10%.

roles of the cysteine residues in the metal binding site, the three point mutants and a double mutant were prepared for analysis using identical conditions as those for the native PR-AMP cyclohydrolase samples (Table 2). Mutation of C93 reduced the Zn²⁺ binding affinity so that only trace levels of the metal were detected upon purification of the protein from *E. coli*. In contrast, the single point mutants C109A and C116A showed either a minor reduction or no change in the protein's Zn²⁺ binding capacity. The combined effect of a double mutant C109A/C116A, however, reduced the protein Zn²⁺ binding affinity so that only trace levels of Zn²⁺ were purified with the protein.

Each of the four PR-AMP cyclohydrolase cysteine mutants were tested for their reactivity toward DTNB in a fashion similar to that used for the wild type enzyme. The TNB²⁻ release stoichiometry for these reactions with each of the four mutant proteins is summarized in Table 3. No reaction could

Table 3. Stoichiometry of Reactions with DTNB

species	stoichiometry (TNB ²⁻ /subunit)
C93A	0 ^a
C109A	0.30 ± 0.03
C116A	0.64 ± 0.02
C109/116A	0.87 ± 0.08
wild type ^b	1.98 ± 0.15

^aNo change in absorbance was observed. ^bSpecific activity 18 U mg⁻¹, all other species were catalytically inactive.

be detected with the C93A mutant and DTNB under these conditions. In contrast, the C109A, C116A, and C109A/C116A mutants reacted with DTNB to different extents all less than wild type enzyme. These data are most consistent with the C93 being the most reactive cysteine in the wild type enzyme.

Subsequent reactions with DTNB are likely a mixed pathway involving both intra- and intermolecular disulfide exchange with C109 and C116. These results are supported by those data presented in Table 1 for DTNB modification followed by cyanylation of PR-AMP cyclohydrolase. The extent of these disulfide exchange reactions is dependent upon the presence of the other two conserved cysteines, but not Zn²⁺, since the C109A/C116A mutant was devoid of metal.

Kinetics of DTNB Reactions with Native PR-AMP Cyclohydrolase and Cysteine Mutants. Figure 4A shows

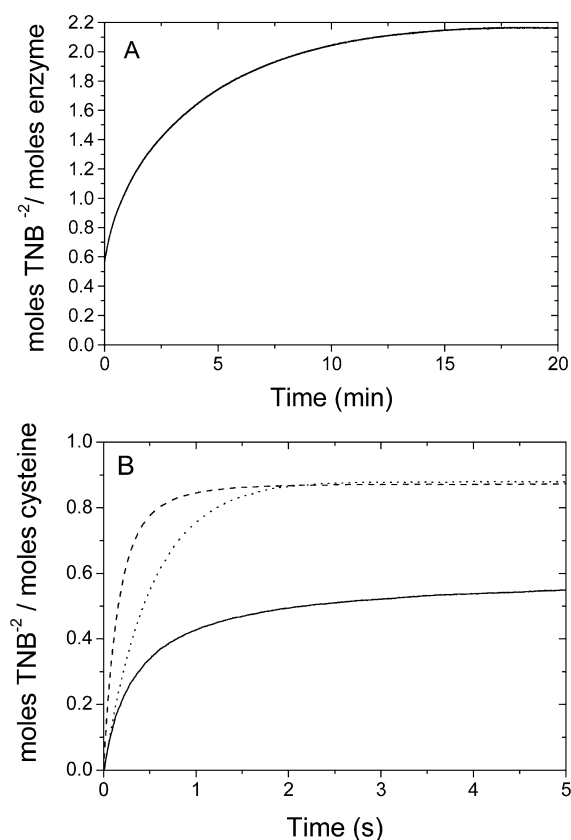


Figure 4. Stoichiometry and kinetics of cyclohydrolase reaction with DTNB. (A) PR-AMP cyclohydrolase complete reaction with DTNB. Conditions 50 mM Tris-HCl pH 8.5, 1 mM EDTA, 5 mM MgCl₂ 30 °C 625 μM DTNB, and 12.5 μM PR-AMP cyclohydrolase. (B) Stopped-flow results for the reaction of DTNB with native PR-AMP cyclohydrolase (solid line), cysteine (dots), and C109/116A (dashes). Conditions 50 mM KH₂PO₄ pH 7.3, 0.5 mM EDTA, 1 mM MgCl₂, 20 °C, 440 μM DTNB, 8.7 μM PR-AMP cyclohydrolase.

the full time course for reaction of native PR-AMP cyclohydrolase with DTNB. The moles of TNB²⁻ anion never exceeded 2 mol per subunit ($\pm 12\%$) in any preparation, even under denaturing conditions. Curve fitting analysis of these progress curves required at least two kinetic phases with an apparent burst phase that accounted for approximately 0.5 mol per subunit modification within 5 s (Figure 4A,B). The rates for the DTNB reaction with native PR-AMP cyclohydrolase at pH 7.3 could be fit to a consecutive reaction model where the first phase corresponded to approximately 1 mol of TNB²⁻ formation per subunit and occurred within 30 s at an apparent first order rate of 246 min⁻¹. The second slower phase of DTNB modification was estimated to be 1000-fold slower (0.24 min⁻¹) and accounted for the second mole of TNB²⁻ formation per subunit.

The C109A/C116A mutant which contains a single cysteine (C93) was used in comparative kinetic studies to analyze the reactivity of this cysteine versus that of the native cyclohydrolase. The kinetics of these reactions with DTNB was compared at two pH conditions. Apparent first-order rate constants for the burst phase of these reactions (1.5 s or less) are summarized in Table 4. Figure 4B also shows the stopped-

Table 4. Stopped-Flow Comparison of DTNB Reactions

source	rate constant ^a (min ⁻¹) pH 7.3	rate constant ^a (min ⁻¹) pH 8.5
wild type	246	930
cysteine	120	324
C109/116A	360	3384

^aApparent first-order rate constants. Conditions as described in experimental except as noted.

flow traces for the native PR-AMP cyclohydrolase and the mutant enzyme. The apparent first-order rate constant of the burst phase with native PR-AMP cyclohydrolase was not significantly different than either C109A/C116A or free cysteine at pH 7.3. However, at pH 8.5, the C109A/C116A demonstrates reaction rate with DTNB that is nearly 10-fold faster than that of free cysteine and 4 fold greater than native PR-AMP cyclohydrolase. In this case, the only residue reacting with DTNB is C93, and there is only trace Zn²⁺ associated with the enzyme (Table 2); the overall stoichiometry of the reaction is 0.87 and is complete within 5 s (Table 3 and Figure 4B). Finally, a C93A mutant did not show any reactivity with DTNB (Table 3) supporting the conclusion that C93 is responsible for the initial fast phase accounting for 0.5 mol of the rapid TNB²⁻ formation upon mixing with the native PR-AMP cyclohydrolase.

Under conditions identical to inactivation, the rate of formation of TNB²⁻ with native PR-AMP cyclohydrolase was followed at 412 nm to compare estimates of the first-order rate constants for inactivation with the appearance of TNB²⁻. The second-order rate constants for the TNB²⁻ formation and inactivation and were estimated at 0.058 μM⁻¹ min⁻¹ and 0.071 μM⁻¹ min⁻¹ respectively, indicating that the overall inactivation and TNB²⁻ formation processes were similar.

Analysis of The Mg²⁺ Binding Site. Conserved residues within the 89–117 protein segments are hypothesized to function as a cocatalytic site. The aspartic acid residues in this sequence are seen to coordinate a cadmium ion in the *M. thermoautotrophicum* hisI crystal structure, suggesting they may bind Mg²⁺, an essential metal for the efficient turnover of the substrate (Figure 1). The protein was also found to undergo an intrinsic change in tryptophan fluorescence upon binding of the product 5'ProFAR; the extent of the fluorescence enhancement was increased 2-fold by inclusion of Mg²⁺ (Supporting Information, Supplemental Figure 4). From the structure of the protein, it is clear that one of the two tryptophans is in the active site and is proposed to be involved in substrate stabilization.²² The roles of these aspartic acid residues, which flank C93 were investigated using site-directed mutagenesis and complementation of the hisI⁻ mutant strain of *E. coli* UTH903. The mutant D94A was not able to maintain viability of the strain, while the mutant D94E was able to perform the necessary hydrolysis step with a 2000-fold reduction in k_{cat}/K_m . A second conservative mutation D92E was also analyzed to show a reduction in k_{cat}/K_m by 175-fold from wild type (Table 2). All aspartate mutants resulted in diminished Zn²⁺ binding of 1.4–1.6 fold per subunit (Table 2). These data support a role for D92 and D94 in properly orienting C93 for Zn²⁺ metal coordination (and by structural implication D96).

CD spectra of native and mutant forms of PR-AMP cyclohydrolase were analyzed to determine if the point mutations and alterations in Zn²⁺ content resulted in significant global conformational changes.⁵⁰ Supplemental Figure 2 shows

spectra of five different PR-AMP cyclohydrolase forms. The spectrum of the native form of the enzyme showed features consistent with both α -helix and β -sheet secondary structural content and this was confirmed by recent structure. As expected, a minimum at 205 nm indicating that the secondary structure contains random coil and antiparallel β -sheet components.⁵¹ Overall, the CD spectra of the three single point mutants were nearly identical to the native PR-AMP cyclohydrolase. These data are consistent with a preservation of the global secondary structure in the mutant enzymes.

Analysis of Stopped Flow Kinetic Data for Possible Intermediate. Previous analysis of the full UV-vis spectrum of this reaction progress has shown the presence of three isosbestic points at 223, 247, and 274 nm respectively. Single turnover reactions were analyzed using stopped-flow analysis of the reaction monitoring from 220 to 310 nm with special attention to the rate of change at the isosbestic points. These data indicated that there are increases and decreases in absorbance consistent with the presence of a detectable intermediate on the path from substrate to product (Supplemental Figure 3). Global fitting of these data indicated the best fits for a two-step model $A > B > C$ with three species including one intermediate (Figure 5 and Supplemental Figure 3). The rates were determined as 23 s^{-1} and 1.4 s^{-1} , respectively, and are consistent with the steady-state k_{cat} of 4 s^{-1} at $30 \text{ }^\circ\text{C}$.

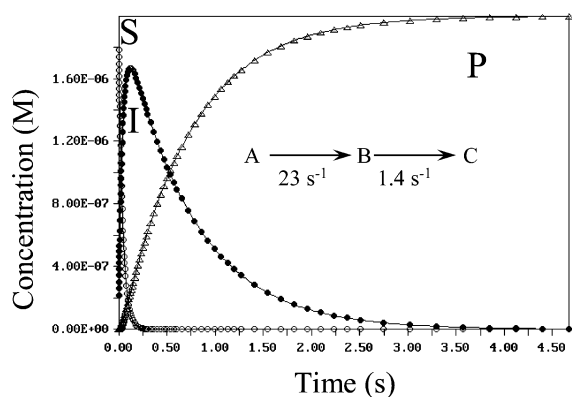


Figure 5. Stopped flow analysis of the PR-AMP cyclohydrolase enzymatic conversion: Plots (averages of triplicate runs) of the time vs concentration for substrate, product, and potential intermediate from global fit of the data to a two-step model $A > B > C$. Open circles (O) are substrate (S), closed circles (●) are intermediate (I), and open triangles (Δ) are product (P) as labeled.

DISCUSSION

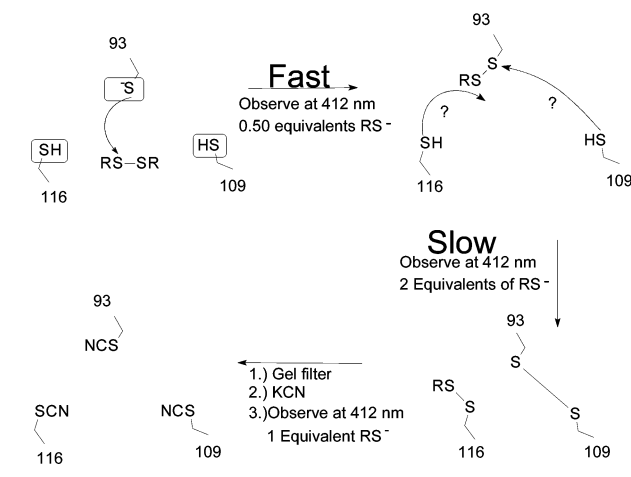
Cysteine Reactivity in PR-AMP Cyclohydrolase. The structure of PR-AMP cyclohydrolase from *M. thermoautotrophicum*²² indicates that the three cysteines, C93, C109, and C116 (C86, C102 and C109 in *M. thermoautotrophicum*), are interacting with a metal ion at the putative active site of the enzyme. The results presented on the chemical modification of native PR-AMP cyclohydrolase establish that these cysteines are accessible to modification by DTNB (and MMTS) and that substrate protects these cysteines from attack. Free Mg^{2+} does not protect PR-AMP cyclohydrolase; in fact it facilitates reaction with DTNB by either directly activating the enzyme or making cysteine more accessible to attack. Evidence from fluorescence binding studies performed with the enzyme

indicated a significant conformational change does take place upon binding substrate in the presence of Mg^{2+} . While MMTS did not show differential reactivity in the presence or absence of exogenous Mg^{2+} , the size differences and relative reactivities of the two reagents are known and their distinctions at the level active site conformation are not unexpected.

Since the cysteine mutants were all catalytically inactive, the reactivity of these cysteines toward DTNB and the metal content were used to assess their roles in metal binding and catalysis. A significant diminution for the reaction of these mutants with DTNB was observed and the presence of C93 was required for reactivity; no attempt was made to further analyze the stoichiometry of the mutant samples by KCN release. However, in the case of the cysteine mutants it was clear that the removal of any cysteine residue resulted in a nonproportional loss of reactivity toward DTNB. This observation is also consistent with the MALDI-MS results for cyanylated enzyme. It is likely that the DTNB label is scrambled after initial attack by the more reactive cysteine and one possible model for these observations can be found in Scheme 2. The amount of TNB^{2-} produced in the reaction

Scheme 2. Potential Model for Cysteine Reactivity Observed with Native PR-AMP Cyclohydrolase

RSSR is DTNB.



with native PR-AMP cyclohydrolase never exceeded 2 mol per subunit ($\pm 12\%$) in any preparation, even under denaturing conditions, indicating either interaction between the cysteines as indicated by Scheme 2 or steric occlusion of the remaining cysteines from reaction. The data further support interpretation of the structural proximity for the cysteines and their functional interdependence at the dimer interface. The reactivity of C93 may be explained by the reduction of its pK_a caused by the flanking aspartate residues.^{52,53}

Zinc Binding and Structure Analysis. Cysteines 93, 109, and 116 are all directly involved in metal binding in the PR-AMP cyclohydrolase active site. The three-dimensional structure of Cd^{2+} -substituted HisI substantiated these findings and implicated that Zn^{2+} ion binding at the subunit interface, with C93 coming from one subunit, while C109 and C116 are from the second subunit. The C93A mutant is completely

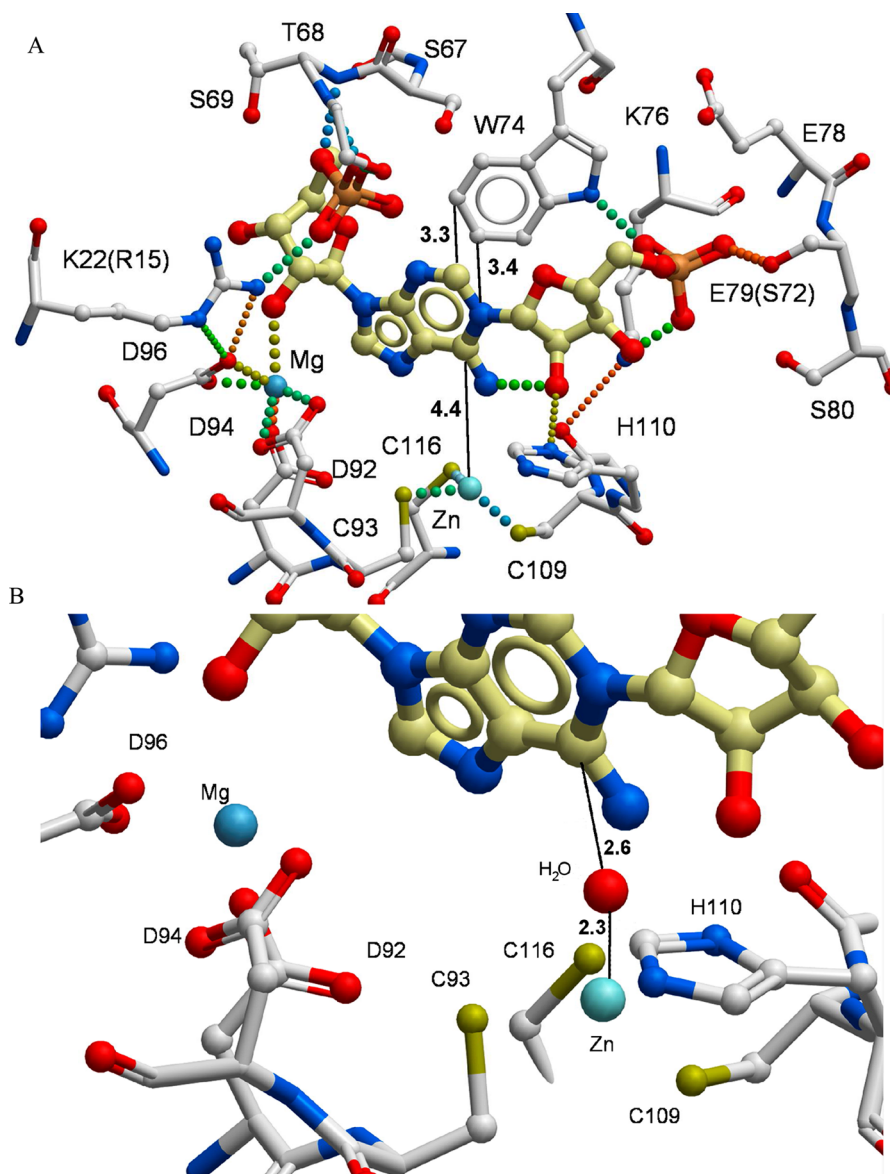


Figure 6. A structural model of the PR-AMP cyclohydrolase active site with substrate docked. (A) The metal Zn^{2+} and Mg^{2+} binding sites and their relationships to the conserved amino acid residues are indicated to assess the functional roles in substrate binding site. The 16 residues labeled in the figure are among the most highly conserved residues described in Figure 1 and reside within 4 Å of the substrate binding site. (B) To better understand the potential positioning of the active site for a zinc-activated water attack on the C6 of the purine ring, a molecule of water was manually positioned into the substrate-docked model. Distances (black dotted lines) are shown in angstroms (Å). Hydrogen bonds and coordinative bonds to metals are rendered as colored spheres.

inactive, and no reaction with DTNB was observed and Zn^{2+} was absent from the protein. The C109A/C116A double mutant was also completely inactive and also lacked the capacity to bind high affinity Zn^{2+} . The single mutants C109A and C116A react partially with DTNB and bind Zn^{2+} to an increasing extent. The presence of Zn^{2+} in the C109A and C116A single mutants influences the reactivity of the second cysteine raising the possibility that the metal increases the relative acidity of these cysteine residues and enhances their reactivity toward disulfide exchange.

Native PR-AMP cyclohydrolase binds a single Zn^{2+} through the three conserved cysteines to function as indicated by the biochemical analysis presented here and is consistent with the Cd^{2+} -substituted crystal structure.²² However, the enzyme is inactive even in the case of C116A where the enzyme has nearly a full complement of zinc demonstrating the importance of all

cysteines to the catalytic activity of PR-AMP cyclohydrolase. It is possible that while C116 is in contact with Zn^{2+} , it is not required for metal binding, unlike C109 and C93, which appear to be at least partially necessary to bind a full complement of zinc. In addition, an invariant H110 resides near these cysteines may also stabilize the site in the absence of one of the other cysteines (except C93). Mutation of H110 to alanine rendered the protein catalytically impaired by a 10^3 reduction in k_{cat}/K_m ; however, this species does bind a full complement of Zn^{2+} .

The crystal structure of *M. thermoautotrophicum* hisI places it in the β -grasp fold family of proteins⁵⁴ and indicates the dimer interface is formed by two beta strands from one monomer ($\beta 1$ and $\beta 3$) packing with $\beta 4$ from the second monomer. At least 1540 \AA^2 or $\sim 40\%$ of each monomer's surface area is buried in the dimer interface.²² The two active sites are located at the dimer interface and appear to have potential for some degree of

cooperativity which is reflected in the differential kinetics of the DTNB reaction.

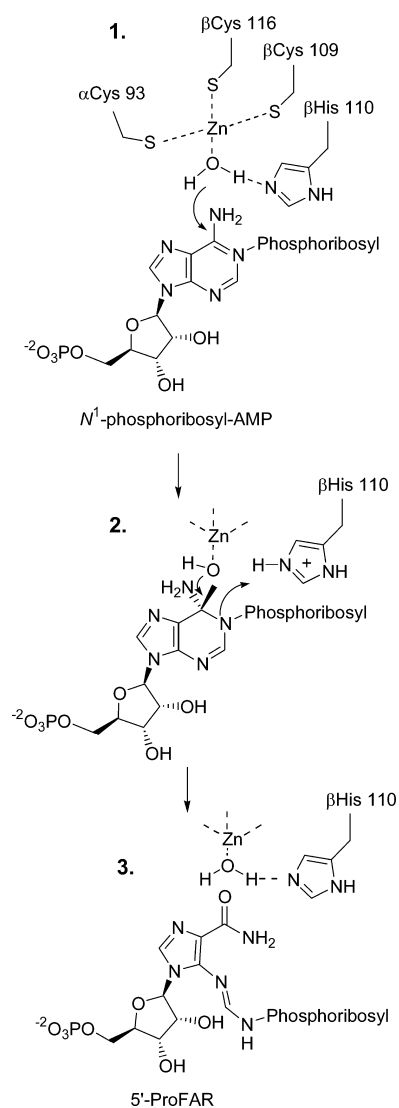
Stabilization of Mg²⁺ Binding. In the structure of PR-AMP cyclohydrolase from *M. thermoautotrophicum*, D92 and D94 together with D96 are ligated to a cadmium ion. The location of this metal could correspond to the magnesium binding site. Complementation and kinetic studies of the mutants of these aspartic acids indicate they have a role in catalysis. Indeed, the k_{cat}/K_m values for conservative mutations were decreased 170-fold for D92E and 2300-fold for D94E. We propose that these aspartates coordinate magnesium, creating a bend in the loop containing C93, which extends this cysteine into the active site. In addition, the Mg²⁺ ion may play a role in substrate binding at the ribosyl ring of PR-AMP. The loss of complementation seen in the alanine mutants of D92 and D94 (and D96; data not shown) indicates an amplified effect in vivo due to the complexity of the environment; suitable levels of magnesium coordination are not achieved in the cellular context reducing substrate binding and catalysis below the threshold for viable histidine biosynthesis.

Substrate Binding Modeling Studies. Upon the basis of the existing protein structure and the additional function data implicating features of the catalytic site, the potential substrate–protein interactions were modeled by docking PR-AMP into the active site. The active site residues were defined by a 4.5 Å radius around the plausible binding site to include 18 key residues of which 16 are invariant and one is functionally conserved among all PR-AMP cyclohydrolases. PR-AMP was imported and docked accounting for the distance required for zinc-activated catalysis. The phosphates were placed and modeled in the oxyanion holes that are defined by conserved residues S67, T68, and S69 for the 5' phosphate and E78, E79, and S80 for the 5'' phosphate. After several manual iterations, the active site and substrate were minimized and several key interactions were observed in the resultant structures (Figure 6A) labeled using *M. vanniellii* numbering (see Figure 1 alignment). Edge to face π stacking between W74 and the adenine ring may assist in substrate binding. The N9 ribosyl group interacts with Mg²⁺ and K22 (R15 in *M. thermoautotrophicum*) resulting in stabilization of the bound substrate. H110 is in a position to π -stack with the incoming substrate, and potentially has a role in catalysis as a nonessential general base. In the volume between the zinc and the purine ring, a water molecule can be positioned (Figure 6B). The proximity of the zinc-activated water to the site of catalysis on the substrate suggests a major role for C93 in the cyclohydrolase reaction. C6 of the substrate in this docked structure would reside within 2.6 Å of a zinc-activated water.

Active Site Architecture Compared to Cytosine Deaminase. The Zn²⁺ metalloenzyme cytosine deaminase from *Saccharomyces cerevisiae* has an active site architecture of functional catalytic groups that has similarities to PR-AMP cyclohydrolase, although the backbone connectivity is distinct. In cytosine deaminase, the Zn²⁺ ligands are cysteine and glutamic acid, while two residues, H62 and W152, interact with the bound inhibitor.¹⁴ Histidine 110 and W74 are invariant residues that reside within highly conserved regions of the protein. The incoming substrate is proposed to be stabilized by interaction with W74 and oriented for catalysis toward the catalytic zinc (Figure 6A). Ligand–metal interactions in both proteins are within 2.5 Å, indicating that while the cysteines in each protein do not superimpose, they are nevertheless in the proper orientation for zinc coordination.

Proposed Mechanism. From the body of experimental and structural data we propose the following mechanism for the activity of PR-AMP cyclohydrolase. The entering substrate is oriented for catalysis by interaction with two residues, W74 and C93. Cysteine 93 interacts with N6 of the purine ring to hold the substrate in the proper orientation for attack by the zinc-activated water. The substrate is bound in the active site through interaction of the N9-phosphoribosyl with the active site magnesium and edge-face π -stacking by W74 with the phosphoribosyl ring at N1. The ring-opening in PR-AMP cyclohydrolase can be deduced from the structural and dynamic studies of cytosine deaminase.^{14,55} Scheme 3 highlights the

Scheme 3. Proposed Catalytic Mechanism for PR-AMP Cyclohydrolase



features of this reaction that are consistent with the current data. The stopped-flow kinetic data revealed the existence of a chemical intermediate on the enzyme with a similar UV extinction coefficient. We interpret these data to be evidence of a tetrahedral intermediate resulting from water addition to the N1–C6 bond of the purine ring system (Scheme 3). The exact stereochemistry of the tetrahedral intermediate cannot be ascertained since inversion is possible at N1 and the lack of constraint on the phosphoribosyl binding interaction not being

heavily anchored in the active site (π -stacking with W74). The attacking water molecule is proposed to be part of the resting enzyme, stabilized by H110, and is activated by the catalytic zinc to attack the purine ring at C6 (Figure 6B). Such a role for histidine has been posited in the structural and mechanistic studies of GTP cyclohydrolase I,⁵⁶ where mutants lacking this histidine were still able to catalyze the cyclohydrolase reaction, similar to our observations. This functional relationship could explain the apparent pK_a 7.3 in the k_{cat}/K_m values as previously described since protonation of the His110 would render it unable to serve as a general base.⁴ In kinetic studies of PR-AMP cyclohydrolase from *M. thermoautotrophicum* we obtained a similar pK_a (7.5) to that of the *M. vanielii* protein whether the active site was populated with zinc or cadmium.²² Protonation of the substrate by the zinc-activated water was suggested as a secondary mechanism in the histidine-deficient mutants. Further distinctions from the cytosine and adenosine deaminase reactions is the fact that ammonia is not produced rather a labile C6–N2 bond is cleaved; protonation of the incipient basic N2 group upon formation of the tetrahedral intermediate renders it a good leaving group. The topological relationship in the enzyme active site that allows for the distinction of N2 and N1 positions is still an open question. The active site of the enzyme is accessible to solvent even when substrate is present and therefore the potential for exchange to facilitate proton transfer is anticipated.

Evolutionary comparisons with the deaminases also indicate some further distinctions from PR-AMP cyclohydrolase. Structural studies of cytosine deaminase from yeast and bacteria indicate this protein underwent convergent evolution, using two separate folds to arrive at the same mechanistic function.^{15,16} Whereas the bacterial isoform utilizes the classical (β/α)₈ barrel, yeast cytosine deaminase has adopted a mixed α/β fold.^{14,15} Each monomer of the PR-AMP cyclohydrolase dimer is composed of two antiparallel beta sheets with a single α helix placing it in the β -grasp fold family.⁵⁴ The structure of PR-AMP cyclohydrolase and the type of metal binding site indicates a functional relationship to other zinc-dependent nucleotide deaminases but with clearly distinct features.⁵⁷ One significant difference in the PR-AMP cyclohydrolase is the use of three cysteine ligands for coordination of the Zn^{2+} metal which also occurs in select other related enzymes.^{7,8,18,57,58} Given the distinctions in the metal binding site ligands, the active site dependence on the dimeric structure, and the additional potential role(s) for Mg^{2+} metal binding sites, a logical proposal emerges that the PR-AMP cyclohydrolase represents a third protein fold that has evolved to function as an amino hydrolase activating water by utilizing zinc.

■ ASSOCIATED CONTENT

● Supporting Information

Supplemental Figure 1: Tris-tricine sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis of purified of mutants. Supplemental Figure 2: CD spectra of aspartate mutants. and WT enzyme. Supplemental Figure 3A–H: stopped-flow evidence for an intermediate in the PR-AMP cyclohydrolase reaction. Supplemental Figure 4: Mg^{2+} fluorescence titration experiments. Supplemental Table 1: MMTS summary of inactivation results. Supplemental Table 2: DTNB Summary of inactivation results. Supplemental Table 3: MALDI-MS results PR-AMP cyclohydrolase and products from cyanilation and cleavage. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

AAS, atomic absorption spectroscopy; CD, circular dichroism spectropolarimetry; ddH₂O, double-distilled deionized water; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, (ethylenedinitrilo) tetraacetic acid; ICP-ES, inductively coupled plasma-emission spectroscopy; HEPES, (*N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]); MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry; MMTS, methyl methane thiosulfonate; NTCB, 2-nitro-5-thiocyano benzoic acid; PCR, polymerase chain reaction; PR-AMP, *N*¹-(5'-phosphoribosyl)adenosine 5'-monophosphate; 5'-ProFAR, *N*¹-[(5'-phosphoribosyl) formimino]-5-aminocarboxamide ribonucleotide adenosine monophosphate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TAE, Tris acetate EDTA; TFA, trifluoroacetic acid; TNB²⁻, nitro-5-thiobenzoate anion; Tris, Tris-(hydroxymethyl)aminomethane

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