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Enzymatic hydrolysis by transition-metal dependent nucleophilic aromatic substitution

Sibel Kalyoncu^{#1}, David P. Heaner Jr.^{#1}, Zohre Kurt², Casey M. Bethel¹, Chiamaka U. Ukachukwu¹, Srinivas Chakravarthy³, Jim C. Spain^{2,4}, and Raquel L. Lieberman^{#1}

¹School of Chemistry & Biochemistry, Georgia Institute of Technology, Atlanta, GA

²School of Civil and Environmental Engineering, Georgia Institute of Technology, Atlanta, GA

³Biophysics Collaborative Access Team, Advanced Photon Source, Argonne National Labs, Lemont, IL

⁴Center for Environmental Diagnostics and Bioremediation, University of West Florida, Pensacola, FL

[#] These authors contributed equally to this work.

Abstract

Nitroaromatic compounds are typically toxic and resistant to degradation. *Bradyrhizobium species* strain JS329 metabolizes 5-nitroanthranilic acid (5NAA), a metabolite secreted by *Streptomyces scabies*, the bacterium responsible for potato scab, an agricultural disease of global economic importance. The first biodegradation enzyme is 5NAA-aminohydrolase (5NAA-A), a metalloprotease family member that converts 5NAA to 5-nitrosalicylic acid. We characterized 5NAA-A biochemically and obtained snapshots of its mechanism. 5NAA-A, an octamer that can use several divalent transition metals for catalysis, employs a nucleophilic aromatic substitution mechanism. Unexpectedly, the metal in 5NAA-A is labile but readily loaded in the presence of substrate. 5NAA-A is specific for 5NAA and cannot hydrolyze other tested derivatives, which are likewise poor inhibitors. The 5NAA-A structure and mechanism expands our understanding of the chemical ecology of an agriculturally important plant and pathogen, and will inform bioremediation and biocatalytic approaches to mitigate the environmental and ecological impact of nitroanilines and other challenging substrates.

Graphical abstract

Correspondence should be addressed to Raquel L. Lieberman, (Raquel.lieberman@chemistry.gatech.edu).

Author Contributions

S.K., R.L.L., D.P.H., J.C.S. designed experiments. S.K., D.P.H., C.M.B., Z.K. conducted enzymatic assays. S.K., D.P.H., R.L.L. solved, refined, and analyzed crystal structures. C.M.B., C.U.U. cloned, expressed, and purified enzymes. S.C. acquired and analyzed SAXS data. R.L.L., J.C.S., S.K., D.P.H. wrote the manuscript.

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Accession Codes

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A vast number of synthetic and natural organic compounds found in the biosphere are poisonous to living organisms^{1,2}. Nitroaromatic compounds, widely synthesized and used industrially for dyes, pesticides, explosives and solvents, are particularly toxic and recalcitrant to degradation. Nitro substituents are deactivating *meta* directors in electrophilic aromatic substitution reactions, and can form a stable Meisenheimer complex when found *ortho* or *para* to a leaving group in nucleophilic aromatic substitution (S_NAr)^{3–5}. Bacteria from areas contaminated with synthetic nitroaromatics have evolved degradation pathways in which the nitro group is first converted to nitrite, amine, hydroxylamine, keto or hydroxyl by oxygenases or nitroreductases; then ring fission leads to intermediates that can enter central metabolism^{3–5}. Naturally occurring nitro-containing compounds are produced in animals, plants, fungi, and microbes^{5–7}, but documentation of their catabolism is scant⁵. Discovery of the enzymatic mechanisms for synthesis and biodegradation of such compounds would extend our understanding of metabolic diversity for the prediction and control of their ecological impacts.

There is growing awareness of the potential use of rhizospheric or endophytic bacteria as biocontrol agents to enhance resistance to plant pathogens^{8–10}, including *Streptomyces scabies*¹¹, which causes potato scab¹², an agricultural disease of worldwide economic importance¹³. The primary phytotoxin, thaxtomin, is a family of 5-nitrotryptophan-containing cyclic dipeptides. Among the additional metabolites secreted by *S. scabies* is 5-nitroanthranilic acid (5NAA, **1**), the nitrated analog of the tryptophan biosynthesis intermediate chorismate. 5NAA is degraded by *Bradyrhizobium* sp. JS329, a bacterial strain isolated from potato field soil¹⁴. The ecological role of *Bradyrhizobium* sp. JS329 is not currently known, but by eliminating a component of the *S. scabies* chemical arsenal, *Bradyrhizobium* sp. JS329 might protect itself and plants from disease^{1,15}. In contrast to the processes known to biodegrade xenobiotic nitroaromatic compounds, the nitro group is not the initial target for the biodegradation of 5NAA. Instead, 5-nitroanthranilate aminohydrolase (UniProtKB/Swiss-Prot: D3WZ85.1, E.C. 3.5.99.8, 5NAA deaminase, 5NAA-A) performs a unique deamination reaction whereby the amino group, which is *para* to the nitro substituent, is hydrolyzed to form 5-nitrosalicylic acid (5NSA, **2**), and the nitro group remains intact. Next, ring fission is catalyzed by 5NSA dioxygenase and the nitro group is eliminated by downstream reactions¹⁶. Whereas DNA deaminases and other hydrolases can remove amino groups from aliphatic and heterocyclic compounds¹⁷, 5NAA-A is the first described hydrolase that can remove an amino group from a substituted benzene.

Here we report the crystal structures of apo 5NAA-A, substrate-bound 5NAA-A, product- and Mn^{2+} -bound 5NAA-A, and the Meisenheimer complex intermediate with Zn^{2+} bound to the 5NAA- $\text{A}_{\text{R}289\text{A}}$ mutant, leading to the proposed nucleophilic aromatic substitution mechanism. We further clarify how 5NAA-A is tailored for its unprecedented and phylogenetically exceptional active site chemistry by comparing enzyme kinetics with different metal ions, investigating 5NAA-A substrate specificity, and providing support for a substrate-assisted mechanism for loading 5NAA-A with its metal ion cofactor.

Results

5NAA-A overall structure and substrate binding site

The crystal structure of apo 5NAA-A (selenomethionine (SeMet) derivatized apo, 2.75 Å resolution; Supplementary Results, Supplementary Table 1), which is notably stable and catalytically competent (Supplementary Fig. 1a,b), confirms inferences from sequence that 5NAA-A belongs to the MEROPS M20 metallopeptidase family¹⁸ and thus evolved from a common ancestor¹⁹. The closest structural homologs are M20 family members: di-zinc carboxypeptidase G2 (root mean squared deviation (R.M.S.D.): 5.04 Å, sequence similarity ~35%, Supplementary Figs. 1c and 2)²⁰, N-acetyl-L-citrulline deacetylase (ACD, R.M.S.D.: 4.32 Å)²¹, and N-succinyl-L,L-diaminopimelic acid desuccinylase (SDAD, R.M.S.D.: 5.66 Å)²² (Supplementary Fig. 2), but 5NAA-A harbors key distinctions leading to its unique supramolecular arrangement and catalytic site (see below).

Analysis of the crystal lattice and small angle X-ray scattering (SAXS) envelope reveal an octamer in which the eight ~43 kDa 5NAA-A protomers are arranged radially around a ~50 Å diameter solvent channel (Fig. 1a, Supplementary Fig. 3) to form a ~350 kDa enzyme. The octamer is unique among characterized M20 family members, which are at most functional dimers²³. A shift in the relative orientation of the two lobes, labeled “catalytic” and “dimerization”²⁰, and novel loop configurations, generate two large surface areas (Supplementary Fig. 2a, b, c). The interface created by the “dimerization” lobe establishes the 5NAA-A catalytic center (see below) and is larger than that of carboxypeptidase, ACD, or SDAD (Fig. 1a, b and Supplementary Fig. 2c). The importance of the dimerization interface is underscored by the fact that the V247K variant, designed to destabilize this interface, could not be purified to appreciable levels (Supplementary Table 2). The interface involving the “catalytic” lobe enables the closed circular arrangement of a tetramer of dimers (Fig. 1a).

The crystal structure of 5NAA-A with bound substrate (5NAA-bound, 3.2 Å resolution, Supplementary Table 1) reveals that 5NAA binds primarily within one catalytic lobe but is stabilized by a key residue, Tyr₂₂₃ from an adjacent monomer, which provides parallel-displaced π - π stacking stabilization (Fig. 1c). Within the catalytic lobe, 5NAA is held in place by Arg₃₇₃ and Arg₂₈₉, which stabilize the carboxyl and nitro groups, respectively, and the side chain of Glu₁₅₈, which is within hydrogen bonding distance of the amino substituent. Asp₁₆₀ and the main chain of Trp₃₇₂ also stabilize the aforementioned adjacent Tyr₂₂₃ (Supplementary Fig. 4a). Alanine variants of Tyr₂₂₃, Arg₂₈₉, and Arg₃₇₃, as well as Y223F, did not generate product or were not isolated in sufficient yield for further characterization (Supplementary Table 2). Two loops disordered in the apo structure are

located in this region of the catalytic lobe. Substrate binding organizes these loops (Fig. 1c), primarily by interactions between Tyr₂₈₈ and Arg₉₈ and between Asp₉₅ and Lys₂₈₆ (Supplementary Fig. 4b).

Metal ion binding site, preferences, and loading

Adjacent to the 5NAA binding site is a triad of residues (His₈₆-Glu₁₉₆-Asn₁₂₄) in a location equivalent to that of the corresponding M20 metalloprotease His₂-Glu₂-Asp metal binding motif. 5NAA-A requires metal ions for hydrolysis, and several transition metal ions are suitable (Fig. 2a,b)¹⁶. Coordinating ligands His₈₆ and Glu₁₉₆ are oriented as in carboxypeptidase, ACD, and SDAD (Supplementary Fig. 2d). Asn₁₂₄, which replaces the Asp found in dinuclear M20 metalloproteases, cannot act as a bridging ligand. At the position of the two remaining ligands that form the second metal binding site in these dinuclear enzymes reside Ile₁₅₉ and Gly₃₈₉ in 5NAA-A (Supplementary Fig. 1c). Neighboring second shell residues such as Asp₈₈ and Glu₁₅₈ provide hydrogen-bonding stabilization to orient the metal binding ligands even in the absence of metal (Supplementary Fig. 4c). Alanine mutants of His₈₆, Glu₁₉₆, Asn₁₂₄, Asp₈₈, Glu₁₅₈, as well as D88N, are not active or result in a misfolded protein that cannot be purified for further characterization (Supplementary Table 2). With 5NAA bound, there are no detectable tunnels or channels leading to the metal center.

Metal loading *in vivo* must be relatively facile given that 5NAA-A is active in clarified cell lysates of *E. coli* (7.7 nmol/min/mg) and *Bradyrhizobium* sp. JS329¹⁴ using the same end point assay¹⁴. Unlike a typical metalloenzyme that has at least nanomolar affinity for metal²⁴, however, biochemical evidence supports the hypothesis that 5NAA-A has weak affinity for metal ions. When sufficient levels of metal ions are included in the reaction mixture (Fig. 2b), kinetic parameters for 5NAA-A are in line with other metabolic enzymes²⁵. Fastest rates are observed in the presence of Mn²⁺ (Michaelis constant (K_m) = 354.0 ± 14.2 μ M, catalytic rate constant (k_{cat}) = 0.605 ± 0.012 s⁻¹) followed by Zn²⁺ (K_m = 133.0 ± 12.9 μ M, k_{cat} = 0.302 ± 0.009 s⁻¹), and Co²⁺ (K_m = 139.6 ± 9.8 μ M, k_{cat} = 0.256 ± 0.006 s⁻¹); Fe²⁺ facilitates turnover but data cannot be fit to the Michaelis-Menten equation (Fig. 2c). Addition of exogenous metal to the purified enzyme for activity *in vitro* is necessary because metal is lost upon purifying recombinant 5NAA-A; incubation with Zn²⁺ or Mn²⁺ does not reconstitute *holo* 5NAA-A (Supplementary Fig. 5a), indicating that metal is labile. In addition, the arrangement of 5NAA-A metal binding residues appears to leave open one or more coordination sites (see below). Indeed, despite optimizing numerous parameters, we could not detect heat of binding for Zn²⁺ or Mn²⁺ to 5NAA-A by isothermal titration calorimetry, (ITC, Online Methods, Fig. 3a and Supplementary Fig. 5b), suggesting that it is below the detection limit. This result is consistent with the high excess metal over enzyme required for activation (Fig. 2b) and in the range expected if the measured K_m approximates the affinity²⁶. Notably, titrating 5NAA or 5NSA alone (Fig. 3b), or a mixture of 5NSA and Mn²⁺ (Fig. 3c), or 5NSA and Zn²⁺ (Supplementary Fig. 5b), into wild-type 5NAA-A reveals low micromolar binding dissociation constants (K_d). No heat of binding is detected upon titrating Mn²⁺ into a solution of 5NAA-A pre-incubated with 5NAA (Fig. 3d), supporting the *in silico* observation that substrate binding occludes metal binding, and is likely due to the organization of two active site loops (Fig. 1c).

In contrast to 5NAA-A, 5NAA has a high apparent affinity for Zn^{2+} based on its facile competition with Indo-1, a sub-nanomolar fluorescent chelator of Zn^{2+} (ref. 27, Supplementary Fig. 5d). The concentrations of free metals in *Bradyrhizobium* sp. JS329 are not known, but total cellular Zn and Mn in *E. coli* and *Bradyrhizobium* sp. JS329 (Supplementary Fig. 5e) are similar to levels reported previously for *E. coli*²⁸; thus, we infer the free metal in *Bradyrhizobium* sp. JS329 is comparable to *E. coli* where free Zn^{2+} is 0.01–0.030 nM²⁹. Cumulatively, our data indicate that metal loading of 5NAA-A *in vivo* is enabled by prior complexation of metal to 5NAA substrate.

Consistent with these biochemical results, all attempts to obtain a structure of metal-loaded 5NAA-A in the absence of substrate or product were unsuccessful, but we solved structures of wild-type 5NAA-A with Mn^{2+} and 5NSA (Mn^{2+} /5NSA-bound, 2.9 Å resolution), and 5NAA-A_{R289A} with Zn^{2+} and 5NAA (Zn^{2+} /Tet-bound, 2.2 Å resolution), which reacted to form the Meisenheimer intermediate (Tet, (*R*)-6-amino-3-(dioxidoazaneylidene)-6-hydroxycyclohexa-1,4-diene-1-carboxylate, **3**, see below and Supplementary Table 1). The two structures confirm the His₈₆-Glu₁₉₆-Asn₁₂₄ metal ligand residues. The structures superimpose well with that of 5NAA-bound; the positions of 5NSA, Tet, and metal binding residues are within coordinate error (Fig. 4a), and there are only minor variations throughout the peptide chain of each of the eight monomers in the asymmetric unit. The coordination geometry for both metals is distorted square pyramidal (Fig. 4a), where the hydroxyl substituent of either 5NSA (Fig. 4b) or Tet (Fig. 4c) occupies the fifth coordination site. Thus, the inferred catalytically competent metal center has octahedral geometry where the fifth position is occupied by the amino group of 5NAA and the sixth coordination site is the water molecule to be activated for hydrolysis.

Proposed catalytic cycle and substrate specificity

On the basis of the available structures, we propose that 5NAA-A-mediated hydrolysis of 5NAA to form 5NSA occurs by $\text{S}_{\text{N}}\text{Ar}$ in a sequence of events that parallels, but is distinct from, metal-mediated proteolysis³⁰ (Fig. 4d). After binding of metal-complexed 5NAA to 5NAA-A, a water molecule from bulk solvent (Supplementary Fig. 1b) is activated by metal coordination to reduce its pK_{a} , and then deprotonated by the general base Glu₁₅₈. Nucleophilic attack of 5NAA at the electrophilic C2 carbon can only occur from a single face, forming the Meisenheimer intermediate with (*R*-) absolute configuration. The now-protonated Glu₁₅₈ stabilizes the hydroxyl and amino groups of the tetrahedral C2 carbon (Fig. 4c). Aromaticity is restored concomitant with the release of the ammonia leaving group to form 5NSA.

5NAA-A exhibits a high level of substrate specificity. 5NAA-A did not catalyze transformations of derivatives including 4-nitroaniline (**4**), 4-amino-3-nitrobenzoic acid (**5**), and 2-bromo-5-nitrobenzoic acid (**6**) when they replaced 5NAA in our enzymatic assay (Supplementary Fig. 6a). ITC studies revealed no detectable binding for **4** or **5** (Supplementary Fig. 6b), and neither **4**, **5**, nor **6** was a competitive inhibitor (Supplementary Fig. 6c). The result for **5** is particularly surprising because it has both functional groups that in 5NAA interact with an arginine. Taken together, the above results indicate that while the electrons in Tet are expected to be primarily delocalized onto the strongly electron

withdrawing *para* nitro group, the *ortho* carboxyl substituent of 5NAA is also important for catalysis.

Identification of 5NAA-A homologs

Phylogenetic analysis of the 5NAA-A protein sequence expands our appreciation for the breadth of non-peptidase M20 homologs lurking among this protease family beyond deacetylases and desuccinylases¹⁸ (Supplementary Data Set and Supplementary Fig. 1c). Though numerous sequences are annotated as M20 peptidase family members, neither the number of metal binding sites in the active site nor biological substrates has been determined experimentally. Manual inspection of sequence alignments of closely related species revealed just one other sequence, *Moorella glycerini* hypothetical protein WP_054938065.1, with the signature residues for both mononuclear metal ion binding and binding of a nitroaniline substrate (Supplementary Data Set and Supplementary Fig. 7). The biological relevance of degradation of 5NAA-like compounds in this thermophilic, anaerobic, endospore-forming bacterium isolated from a hot spring sediment in Yellowstone National Park³¹, is a mystery. Review of other selected sequences revealed several additional related enzymes from endospore-forming bacteria, e.g. *Cohnella thermotolerans* peptidase M20 WP_051318224 and *Nitrospirillum amazonense* peptidase M20 WP_004272836.1, which have the same 5NAA-A metal binding residues and may hydrolyze aromatic substrates (Phe in position of Tyr₂₂₃) harboring a carboxylate (Arg in position Arg₃₇₃), but are unlikely to stabilize a nitro group (Gly or Ala in place of Arg₂₈₉) as in 5NAA-A (Supplementary Fig. 7).

Discussion

5NAA-A has undergone key adaptations to enable hydrolysis of 5NAA while protecting the integrity of the nitro substituent. By modifying the bi-lobed M20 dinuclear metalloprotease architecture with key new side chain residues, mostly notably Arg₂₈₉, Arg₃₇₃, and Tyr₂₂₈ from an adjacent monomer, plus three ligands for a mononuclear metal ion binding site within each polypeptide chain, the 5NAA-A octamer lowers the large energetic barrier to catalysis, activates a water molecule and the C2 carbon on 5NAA for nucleophilic attack, and stabilizes the Meisenheimer complex to form 5NSA. Even though the hydrolytic chemistry of 5NAA-A involves a high energy intermediate where aromaticity has been broken, the enzymatic reaction occurs under physiological conditions. The chemistry of 5NAA-A is distinct from unrelated DNA deaminases¹⁷; both enzymes activate water via a mononuclear metal ion site, but different ligands, coordination environments, and active site residues accommodate the purine/pyrimidine and corresponding reactive intermediates. The metal dependence of 5NAA-A renders it also distinct from glutathione-S-transferase and 4-chlorobenzoyl-CoA dehalogenase, the two other enzymes proposed to undergo S_NAr³².

A single divalent transition metal ion cofactor is required for catalysis, but 5NAA-A neither has a high affinity metal binding site nor obvious preference for a particular ion *in vitro*. Among the activating first row transition metals ions, Michaelis Menten kinetic behavior is observed for Mn²⁺, Zn²⁺, and Co²⁺, but not Fe²⁺, and 5NAA-A is not activate with Ni²⁺ or Cu²⁺. Given the similarity in pK_a values for these metal ions³³, water activation should be

similar across these metals so perhaps catalytic rates are dictated by geometric features and electronic properties suitable for activating 5NAA for attack and stabilizing the Meisenheimer intermediate.

Our data indicate that metal loading in 5NAA-A is substrate-assisted. The operon for 5NAA degradation does not include a metallochaperone-encoding gene¹⁴, supporting the proposal that there is no specialized process for delivering metal to 5NAA-A. While unusual, enzymes involving nucleotide cofactors or substrates, e.g. kinases³⁴ or DNA polymerase³⁵ function this way. These enzymes require a metal ion for catalysis, typically Mg^{2+} , have a defined metal binding site in the enzyme though no measurable affinity³⁶, and metal is loaded as a complex with the nucleotide. The coordination chemistry of 5NAA with metal ions, particularly complexes competent for loading to 5NAA-A and hydrolysis, remains unexplored. We speculate that the electron withdrawing nitro group will render the *para* amino substituent a weak ligand, so metal ion complexation in solution will involve the carboxylate ligand, which will be handed off to Arg₃₇₃ and enable reorganization to the observed substrate and metal configuration within the 5NAA-A active site.

Further studies are required to evaluate the extent of promiscuity of 5NAA-A in metal ion utilization *in vivo*, but the scenario in *Bradyrhizobium* sp. JS329 is reminiscent of the Mn-dependent antioxidant strategy employed by other microbes³⁷. The relative flexibility of 5NAA-A in metal utilization may be a solution to enable hydrolysis even with fluctuations in 5NAA levels or bacterial metal content. Levels of soil nutrients, including Mn^{2+} salts, are known to affect the formation of potato scab³⁸, so it is possible that 5NAA complexation with metal occurs prior to cellular uptake. In support of this notion, metal coordination may protect the electrophilic 5NAA from intracellular nucleophiles like glutathione, which likely form adducts that are not 5NAA-A substrates. Once transported intracellularly, 5NAA may remain bound to the same metal ion or exchange for one preferred for hydrolysis by 5NAA-A.

Finally, 5NAA-A expands our knowledge of the largely-uncharacterized metalloproteome³⁹. 5NAA-A would not have been identified as a transition metal-dependent enzyme by proteome-scale studies, which follow metal content during purification. Whereas bioinformatics correctly predicts 5NAA-A to be a metal dependent enzyme, our findings reinforce the challenges associated with functional annotation of sequences from large families in the absence of known biological substrates⁴⁰. We identified additional M20 non-peptidase homologs that likely harbor labile mononuclear metal binding sites and hydrolyze other aromatic substrates of potential environmental or synthetic interest. It is not possible to identify substrates from the sequence or structure, but it appears likely that many additional rare and unusual enzymes are involved in catabolic pathways for the great diversity of natural organic compounds in our ecosystem².

Online Methods

Molecular Biology

5NAA-A was cloned into the pET-30 Xa/LIC vector (Novagen). 5NAA-A variants were generated by site-directed mutagenesis (QuickChange Lightning Kit, Agilent Technologies)

using the 5NAA-A pET-30 Xa/LIC vector as template. Primers were designed with the assistance of PrimerX (Bioinformatics.org), and synthesized by MWG Operon. Sequences of all the constructs were confirmed by DNA sequencing (MWG Operon). Primer sequences are listed in Supplementary Table 2.

Protein Expression and Purification

Wild-type and variants of 5NAA-A were expressed in *E. coli* Rosetta 2 (DE3) cells. A single transformed colony was inoculated into a selective 20 mL Luria-Bertani culture (LB, Fisher, supplemented with 34 µg/mL chloramphenicol, 50 µg/mL kanamycin) and agitated overnight (16–18 hours) at 37°C, 225 RPM. A starter culture (5 mL) was used to inoculate 1 L of selective LB media and agitated at 37°C, 225 RPM until an optical density at 600 nm of 1.0–1.5 was reached. The cell cultures were cooled with shaking at 225 rpm at 25°C for 1 hour, then induced with 0.5mM isopropyl β-D-thiogalactopyranoside (IPTG, Calbiochem), and allowed to grow overnight (16–18 hours) at 25°C, shaking at 225 RPM. Cells were pelleted (3000 × g, 10 minutes, 4°C) and subsequently flash-frozen with liquid nitrogen and stored at –80°C.

SeMet derivatized apo 5NAA-A was prepared by transforming the pET-30 Xa/LIC plasmid into competent *E. coli* T7 Express Crystal cells (New England Biolabs). A single transformed colony was inoculated into 10 mL selective minimal media (5x M9 salts, 0.4% glucose, 1 mM MgSO₄, 0.1 mM CaCl₂, 0.0002% ferric ammonium citrate, 34 µg/mL chloramphenicol, 50 µg/mL kanamycin) containing 50 µg/mL L-methionine. The starter culture was agitated at 225 RPM at 37°C for 12 hours, followed by inoculation at a 1:100 dilution into selective 1 L minimal media containing 50 µg/mL L-methionine. When an optical density at 600 nm of 0.6–0.8 was reached, cells were pelleted and resuspended into fresh selective minimal media with no L-methionine and shaken at 225 RPM, 37°C, for 2.5 hours. L-selenomethionine was then added to the cell culture to a final concentration of 50 µg/mL; after 30 minutes with agitation, cells were induced with 0.5 mM IPTG, and then allowed to grow overnight (16–18 hours) at 25°C, with 225 RPM agitation. Cells were pelleted by centrifugation (3000 × g, 10 minutes, 4°C) and subsequently flash frozen with liquid nitrogen and stored at –80°C.

Cell pellets were thawed and suspended in Ni²⁺-affinity purification wash buffer (50 mM HEPES pH 7.5, 500 mM NaCl, 40 mM imidazole, 10% glycerol) containing 0.5x Roche Complete EDTA-free Protease Inhibitor and lysed by two passages through a French Press (13000 psi). Cellular debris was removed by ultracentrifugation (162,000 × g, 45 minutes, 4°C). The supernatant loaded onto a 1 mL Ni²⁺-affinity purification column (GE Healthcare) on an AKTA FPLC system (GE Healthcare), equilibrated with wash buffer. Pure protein was eluted with a gradient from 40–500 mM imidazole. Fractions containing pure protein were concentrated using an Amicon 10K MWCO filter (EMD Millipore) and exchanged in Factor Xa cleavage buffer (50 mM Tris pH 7.5, 100 mM NaCl, 5 mM CaCl₂). The N-terminal hexahistidine tag was removed by incubation with Factor Xa (Roche, 50:1 mass ratio) for 20 hours at 4°C. The digestion mixture was applied to a re-equilibrated 1 mL Ni²⁺-affinity purification column, the flow-through fractions concentrated in an Amicon 10K MWCO filter, and the concentrate then loaded onto a HiLoad 16/600 Superdex 75 prep grade column

(GE healthcare) equilibrated with gel filtration buffer (50 mM Bicine pH 7.0, 150 mM NaCl). The gel filtration buffer was selected by assessing 5NAA-A thermal stability in a range of buffers with various pHs using differential scanning fluorimetry as described previously⁴¹. The fractions containing 5NAA-A were concentrated in an Amicon 10K MWCO filter for further use. Protein purity was assessed by 12% SDS-PAGE analysis with Coomassie staining. Protein concentrations were measured by absorbance at 280 nm using molar extinction coefficient ($74,461.88 \text{ M}^{-1} \text{ cm}^{-1}$) determined by total amino acid analysis (Molecular Structure Facility, University of California, Davis) and molecular weight (42,832.31 g/mol). The molecular mass of purified 5NAA-A was estimated using a Superdex-200 10/300 GL column (GE Healthcare) equilibrated with 5NAA-A gel filtration buffer and calibrated using a gel filtration calibration kit (GE Healthcare).

Circular Dichroism (CD)

CD spectra and thermal melts were acquired on a Jasco J-810 spectropolarimeter equipped with Neslab RTE 111 circulating water bath and a Jasco PTC-4245/15 temperature control system. CD spectra of gel filtration buffer (blank) and $7.8 \mu\text{M}$ WT 5NAA-A sample in gel filtration buffer were measured at room temperature using a 0.1 cm cuvette. CD thermal melt was performed utilizing a $1^\circ\text{C}/\text{min}$ increase in temperature from 5 to 91°C . After blank-subtraction, data were converted to molar ellipticity $\Theta = (\Theta_{\text{obs}} \times 106)/(\text{pathlength (mm)} \times c \times n)$, where Θ_{obs} is the observed ellipticity (mdeg) at wavelength λ ; c is the protein or peptide concentration (μM); and n is the number of residues. Fifteen scans were averaged from 300 to 200 nm at a 200 nm/min scan rate. The melting temperature was determined using ellipticity values recorded at 220 nm via sigmoidal dose-response analysis in GraphPad Prism 5 software.

Activity and Inhibition Assays

The chemical structures of commercial 5NAA (Sigma Aldrich) and 5NSA (Eastman) were confirmed by ^1H -NMR (Georgia Tech NMR Center).

To assess the activity of 5NAA-A during purification steps, an analytical high-performance liquid chromatography (HPLC) assay¹⁴ was performed on a sample (a) after ultracentrifugation, (b) after elution from first Ni^{2+} -affinity column, (c) after the second Ni^{2+} -affinity purification (flow-through), and (d) after gel filtration. 5NAA and 5NSA content was analyzed by HPLC with an Agilent Eclipse XDB-C18 column (4.6 mm by 150 mm) by the method described previously¹⁴.

A spectrophotometric assay was developed to follow depletion of substrate (λ_{max} for 5NAA= 374 nm; λ_{max} for 5NSA= 306 nm). Divalent transition metals (MnSO_4 , $\text{Zn}(\text{OAc})_2$, CoCl_2 , CdCl_2 , NiSO_4) and alkali metals (MgCl_2 , CaCl_2) were screened for 5NAA-A activity. 5NAA (100 μM) and varying concentrations of metals (0.25 μM , 2.5 μM , 50 μM , 500 μM) were dispensed in a 96-well plate (Costar 96-Well EIA/RIA). Freshly purified 5NAA-A enzyme (0.25 μM) in gel filtration buffer was added (100 μL total volume) and the reaction mixture was mixed on ice by gentle pipetting. The plate was sealed with optical adhesive film (Micro-Amp) and the reaction was initiated in a BioTek Synergy 2 plate reader

set to 37°C. Absorbance readings were recorded every 3 minutes for 2 hours. 5NAA depletion (374 nm) was used for data analysis.

For Michaelis-Menten assays, a range of enzyme concentrations (0.15, 0.25, 0.5 μM) were initially tested, and 0.25 μM was selected for further assays. Varying concentrations of 5NAA (25–600 μM) and 500 μM MnCl_2 , $\text{Zn}(\text{OAc})_2$, $\text{Cu}(\text{OAc})_2$, CoCl_2 , were distributed and the same procedure above was performed. Due to its oxygen sensitivity, assays with Fe^{2+} were conducted in a Coy anaerobic chamber (95% Ar, 5% H_2). Solutions of gel filtration buffer (5 ml), 5NAA solution (2.5 ml), and 5NAA-A (0.15 ml) were degassed at room temperature by sparging Argon gas for ~10 minutes. $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ (EM Science) was dissolved in deoxygenated water under anaerobic conditions (10 mM stock solution). Plate reaction contents were dispensed, mixed, and the plate sealed at room temperature in the anaerobic chamber. The plate was removed and the assay measured as described above for 30 minutes. For all experiments, absorbance readings of solutions containing all reagents except enzyme were subtracted from those containing enzyme to obtain the signal for 5NAA depletion.

Data were fit using the Michaelis–Menten analysis option within GraphPad Prism 5. The Michaelis–Menten equation is $V = V_{\text{max}}[\text{S}] / (K_{\text{m}} + [\text{S}])$ where V is the initial velocity, V_{max} is the maximum velocity at infinite substrate concentration, $[\text{S}]$ is substrate concentration, and K_{m} is the substrate concentration at half maximal velocity. Initial velocities were determined by the absorbance reading at 374 nm over the first 15–20 min (negative slope of linear regression). Standard calibration curves were plotted with substrate controls (0–600 μM) for each assay, then initial velocities were plotted against substrate concentrations. Nine replicates were performed for 5NAA-A with MnCl_2 , $\text{Zn}(\text{OAc})_2$, six replicates for 5NAA-A with and CoCl_2 , three for $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$, and six replicates for all 5NAA-A mutants with MnCl_2 or $\text{Zn}(\text{OAc})_2$. Data for Cu^{2+} and Fe^{2+} data did not fit to a Michaelis-Menten model (see main text). Results are reported as mean $\pm$ SEM.

The ability of 5NAA-A to hydrolyze the substrate analogs **4** (Sigma Aldrich) dissolved in water, **5** (Acros) dissolved in 50% (v/v) DMSO, and **6** (Alfa Aesar) dissolved in 100% (v/v) DMSO, was monitored by a discontinuous enzyme activity assay. In an eppendorf tube, 0.25 μM 5NAA-A, either 500 μM MnSO_4 or MnCl_2 , and 500 μM 5NAA (positive control, adjusted to highest final concentration of DMSO used, ~10%), **4**, **6**, or 600 μM **5** were mixed in gel filtration buffer. The reaction was stopped after overnight incubation (16 hours in a 37°C water bath) by the addition of 10% (v/v) trifluoroacetic acid. UV-Vis spectra (300–500 nm) of the reaction mixtures were measured after initial set up and again after quenching using by a BioTek Synergy 2 plate reader to detect any depletion of the respective substrate analog.

To test for competitive inhibition purified 5NAA-A (0.25 μM) was kept constant and concentrations of **4**, **5**, or **6** were varied from 0.125 μM (0.5x enzyme) to 1000 μM (4000x enzyme). Enzyme, 500 μM $\text{Zn}(\text{OAc})_2$ and corresponding analogs were incubated at room temperature for 30–60 min. 5NAA substrate (100 μM) was added and the reaction started as described previously. DMSO at the highest levels used for **5** and **6** were added to reactions

with 5NAA-A with 5NAA as vehicle controls. Data were fit by the half maximal inhibitory concentration (IC₅₀) protocol within GraphPad Prism 5.

Crystallization, X-ray Data Collection, and Structure Determination

Conditions for crystallization of 5NAA-A were initially identified by sparse matrix screening (Hampton Index HT and Rigaku Wizard 1 & 2) and further optimized. SeMet apo 5NAA-A crystals (9.4 mg/mL protein in 50 mM Bicine pH 7.0, 150 mM NaCl) were grown by the sitting-drop method at room temperature in 1.07 M NaH₂PO₄, 0.83 M K₂HPO₄. Crystals of 5NAA-A (7 mg/mL protein in 50 mM Bicine pH 7.0, 150 mM NaCl) with 5NAA (0.22 mM) were grown by the sitting-drop method at room temperature in 1.27 M NaH₂PO₄, 0.63 M K₂HPO₄. Crystals of 5NAA-A (5.8 mg/mL protein in 50 mM Bicine pH 7.0, 150 mM NaCl) with MnCl₂ (16 mM) and 5NSA (0.16 mM) were grown by sitting-drop method at room temperature in 0.1 M sodium cacodylate pH 6.5, 0.75 M trisodium citrate. Crystals of 5NAA-A_{R289A} (5.8 mg/mL protein in 50 mM Bicine pH 7.0, 150 mM NaCl) with Zn(OAc)₂ (12 mM) and 5NAA (0.22 mM) were grown by sitting-drop method at room temperature from a solution containing 0.1 M sodium cacodylate pH 6.5, 0.65 M trisodium citrate.

Crystals of SeMet apo 5NAA-A, 5NAA-A with 5NAA, 5NAA-A with Mn²⁺/5NSA, and 5NAA-A_{R289A} with Zn²⁺/5NAA were cryo-cooled in a solution containing the corresponding reservoir solution supplemented with 10% ethylene glycol, 16.6% glycerol, 23% glycerol, and 20.5% glycerol, respectively. Diffraction data were collected at beamline 22-ID of the Southeast Regional Collaborative Access Team (SER-CAT) at the Advanced Photon Source, Argonne National Labs. Data were indexed, integrated, scaled and processed using HKL2000⁴². The initial structure (5NAA-A SeMet apo) was solved by Se anomalous phasing using AutoSol⁴³ and the remaining structures (5NAA-A with 5NAA, 5NAA-A with Mn²⁺/5NSA and 5NAA-A R289A with Zn²⁺/5NAA) by molecular replacement using Phaser⁴⁴, using a monomer from the SeMet apo 5NAA-A structure as a search model. The models were iteratively built and refined using Coot⁴⁵ and Phenix.refine⁴⁶. eLBOW⁴⁷ within Phenix was used to generate ligand restraints of 5NAA, 5NSA, and the 5NAA intermediate (Tet, (*R*)-6-amino-3-(dioxidoazanylidene)-6-hydroxycyclohexa-1,4-diene-1-carboxylate) from coordinates generated by the assistance of ChemDraw. Readyset⁴⁶ within Phenix was used to generate metal coordination restraints. With the exception of two loops in apo 5NAA-A (residues 91–108 and 393–397), polypeptide chains were readily traced from residues 6–425; electron density for the remaining N-terminal residues varied among monomers and structures. 5NSA was modeled in all eight protomers in the Mn²⁺/5NSA structure. Tet was only well-resolved in six protomers of 5NAA-A_{R289A} crystallized with Zn²⁺/5NAA. Water molecules are only modeled in the 2.2 Å resolution structure where they were visible. Crystallographic statistics for all four structures appear in Supplementary Table 1.

Mass Spectrometry

The conversion of 5NAA to 5NSA by 5NAA-A was confirmed by mixing 0.25 μM 5NAA-A, 500 μM MnCl₂, and 200 μM 5NAA in a total of 200 μL of which 154 μL was H₂¹⁸O (Cambridge Isotope Laboratories, Inc.). The reaction was incubated at 37 °C for 18 hours

prior to mass spectrometry analysis at the Georgia Institute of Technology Bioanalytical Mass Spectrometry Facility by liquid chromatography- mass spectrometry with a C18 reversed phase column coupled to an LTQ Orbitrap XL mass spectrometer (Thermo Scientific).

To measure enzyme activity of crystals, approximately 50 wild-type, apo 5NAA-A crystals grown as above (crystallization solution 1.07 M NaH₂PO₄, 0.83 M K₂HPO₄) were harvested after 3d. The crystal assay solution consisted of a 1:1 mixture of the crystallization solution and 700 μ M 5NAA and 500 μ M MnCl₂ prepared in gel filtration buffer. After washing crystals by brief transfer to crystallization solution (4 μ l), the crystals were added to 10 μ l of the crystal assay solution. After 1 hr incubation at room temperature, the solution was centrifuged at 8000 $\times$ g for 5 min to sediment the crystals, and the supernatant analyzed by mass spectrometry as above.

ITC Measurements

5NAA-A was prepared in gel filtration buffer at 6.4 μ M in 1.8 ml aliquots. Stock solutions of 1 mM 5NAA, 1 mM 5NSA, 3.2 mM **4**, 3.5 mM **5**, 100 mM Zn(OAc)₂ and 100 mM MnCl₂ were diluted to 0.15 mM with gel filtration buffer accordingly. All samples were degassed on a vacuum line (MicroCal ThermoVac) before ITC measurements (MicroCal VP-ITC). In a typical experiment, an automated sequence of 30 injections of 9 μ L of sample was applied to the protein in the sample cell with 6 min between injections to allow for equilibration. The reaction solution was stirred at 270 RPM, and the temperature of the chamber was maintained at 25°C. Several ITC parameters were varied in an attempt to detect a heat of binding of metal ion to 5NAA-A, including changing the enzyme concentration (7.8 μ M), metal ion concentration (250, 500 μ M), salt (e.g. ZnCl₂), and for the experiment in which 5NAA-A was preincubated with 5NAA, time of incubation (1 h).

Data were analyzed with the Origin 7.0 software package from MicroCal, using one-site binding model. A background correction was applied to each experiment by subtracting the average of the last four injections. Background corrections were in accordance with control experiments (gel filtration buffer). A non-linear least squares method was used to obtain one-site model best fit parameters for the number of binding sites, *n*, the association constant, *K_a*, and the change in enthalpy, ΔH° . The dissociation constant, *K_d* was calculated by taking the inverse of *K_a*. Calculated parameters are mean $\pm$ standard deviation.

Competition of 5NAA and Indo-1 for Zn²⁺

The fluorescent chelator Indo-1 (Biotium, 1 μ M prepared in water) was mixed with triplicate preparations of 0–100 μ M Zn(OAc)₂ in 10 mM phosphate pH 7.2, 200mM NaCl (PBS) and incubated in the dark at room temperature for 10 min. Fluorescence emission spectra (350 – 600 nm; λ_{ex} = 338 nm) were acquired for each concentration of Zn(OAc)₂ using a BioTek Synergy H4 Multi-Mode Plate Reader. Values at λ_{max} = 453 nm were plotted against Zn(OAc)₂ concentration to demonstrating saturation for Zn(OAc)₂ above 50 μ M. For competition with 5NAA, 100 μ M Zn(OAc)₂ was mixed with 1 μ M Indo-1 in PBS and incubated in the dark at room temperature for 10 minutes. Aliquots corresponding to final concentrations of 0–650 μ M 5NAA were added and triplicate samples incubated in the dark

for another 10 minutes. Fluorescence readings ($\lambda_{\text{ex}} = 338 \text{ nm}$, $\lambda_{\text{em}} = 453 \text{ nm}$) were acquired. Fluorescence emission at 453 nm was plotted against 5NAA concentration using GraphPad Prism 5.

Total Reflection X-ray Fluorescence (TXRF) measurements

TXRF measurements were performed with a Bruker S2 Picofox TXRF instrument using quartz glass disks (Bruker Instruments). The excitation settings were 50 kV and 750 mA. Measurements were performed by signal integration over 1000 seconds. Quantification of the analytes was performed by Bruker S2 Picofox TXRF software. After overnight dialysis of apo 5NAA-A against either Mn^{2+} or Zn^{2+} (50x excess of protein concentration) by 10K Slide-A-Lyzer Dialysis Cassette, the protein solution was re-run on a Superdex-200 10/300 GL column equilibrated with gel filtration buffer to remove excess metal. Such experiments were performed twice. Quantification of the metal:protein ratio was based on the known internal standard gallium (1 ppm) and 5NAA-A quantity. For determination of total Mn and Zn in *E. coli* and *Bradyrhizobium* sp. JS329 cell lysates, first cells of *E. coli* were grown as above but without IPTG induction, and cells of *Bradyrhizobium* sp. JS329 were cultured without 5NAA¹⁴. Equivalent wet cell mass (1 g) for each bacterium culture was resuspended in buffer (12.5 ml), lysed by French Press and clarified by ultracentrifugation as described above. Total protein contents were similar as indicated by comparable absorbance at 280 nm (30 mg/ml). Clarified lysate was flash-frozen and stored at -80°C until TXRF analysis. A standard curve for MnCl_2 and ZnCl_2 was generated by linear regression of TXRF values obtained for 0.1, 10, 1000 μM MnCl_2 and ZnCl_2 solutions ($R^2 = 0.99$ for both). The standard curve equation was then used to calculate Mn/Zn concentrations for clarified lysates; no Mn or Zn was detected in buffer used for lysis.

Size-Exclusion Chromatography - Small Angle X-ray Scattering (SEC-SAXS)

SEC-SAXS experiments were performed at BioCAT (beamline 18ID, Advanced Photon Source at Argonne National Labs). The camera included a focused 12 KeV ($1.03 \text{ \AA}$) X-ray beam, a 1.5 mm quartz capillary sample cell, a sample to detector distance of $\sim 3.5 \text{ m}$, and a Pilatus 3 1M detector (Dectris). The q -range sampled was $\sim 0.0042\text{--}0.38 \text{ \AA}^{-1}$. In order to ensure sample monodispersity, we used an in-line SEC setup, which included an AKTA FPLC system with a Superdex-200 Increase 10/300 GL column (GE Healthcare). The elution trajectory after the UV monitor was redirected to the SAXS sample flow-cell. Exposures (1 second) were collected every 2 seconds during the gel-filtration chromatography run. Appropriate exposures were averaged and used as the buffer curve for each run, and the exposures during elution (co-incident with the UV peak on the chromatogram) were treated as protein+buffer curves. Data were corrected for background scattering by subtracting the buffer curve from protein+buffer curves. Radius of Gyration (R_g) was calculated using Guinier approximation and a $P(R)$ curve, both of which were done using PRIMUS⁴⁸. D_{max} was also calculated using the $P(R)$ curve and the output was then used to calculate molecular envelopes using DAMMIN 9 separate times⁴⁹. These envelopes were then averaged using DAMAVER and DAMFILT⁵⁰. The high resolution crystal structure was fit into the envelope using SUPCOMB⁵¹.

In Silico Sequence and Structure Analysis

Sequence alignments were conducted using CLUSTAL-Omega⁵². Comparison to known structures in the PDB was performed using BioXGEM⁵³. Interfaces were calculated with PDBePISA⁵⁴. Tunnels and channels were evaluated in CAVER⁵⁵, homology models were generated in SWISS-MODEL⁵⁶, and computational solvent mapping to identify binding sites for small molecules was conducted with FTmap⁵⁷. Evolutionary analyses were conducted in MEGA6⁵⁸. The 5NAA-A sequence was queried against the non-redundant protein sequence database, allowing for up to 20,000 target sequences. The resultant 2,256 sequences were then aligned using MUSCLE⁵⁹. The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model⁶⁰ and the tree with the highest log likelihood (-372632.2194) is presented. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 268 positions in the final dataset. All structure figures were generated in PyMOL (www.pymol.org). Unless stated otherwise, interaction distances are reported for a representative protomer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. McGuinness M, Dowling D. Plant-associated bacterial degradation of toxic organic compounds in soil. *Int. J. Env. Res. Pub. He.* 2009; 6:2226–2247.
2. Davies J. Small molecules: The lexicon of biodiversity. *J. Biotechnol.* 2007; 129:3–5. [PubMed: 17208324]
3. Spain JC. Biodegradation of nitroaromatic compounds. *Annu. Rev. Microbiol.* 1995; 49:523–555. [PubMed: 8561470]
4. Symons ZC, Bruce NC. Bacterial pathways for degradation of nitroaromatics. *Nat. Prod. Rep.* 2006; 23:845–850. [PubMed: 17119634]
5. Ju KS, Paraless RE. Nitroaromatic compounds, from synthesis to biodegradation. *Microbiol. Mol. Biol. Rev.* 2010; 74:250–272. [PubMed: 20508249]
6. Parry R, Nishino S, Spain J. Naturally-occurring nitro compounds. *Nat. Prod. Rep.* 2011; 28:152–167. [PubMed: 21127810]
7. Winkler R, Hertweck C. Biosynthesis of nitro compounds. *ChemBioChem.* 2007; 8:973–977. [PubMed: 17477464]

8. Kobayashi A, Kobayashi YO, Someya N, Ikeda S. Community analysis of root-and tuber-associated bacteria in field-grown potato plants harboring different resistance levels against common scab. *Microbes Environ.* 2015; 30:301–309. [PubMed: 26657303]
9. Compant S, Duffy B, Nowak J, Clement C, Barka EA. Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. *Appl. Environ. Microbiol.* 2005; 71:4951–4959. [PubMed: 16151072]
10. Diallo S, et al. Mechanisms and recent advances in biological control mediated through the potato rhizosphere. *FEMS Microbiol. Ecol.* 2011; 75:351–364. [PubMed: 21204870]
11. King RR, Lawrence CH, Calhoun LA. Unusual production of 5-nitroanthranilic acid by *Streptomyces scabies*. *Phytochemistry.* 1998; 49:1265–1267.
12. King RR, Calhoun LA. The thaxtomin phytotoxins: sources, synthesis, biosynthesis, biotransformation and biological activity. *Phytochemistry.* 2009; 70:833–841. [PubMed: 19467551]
13. Lerat S, Simao-Beauvoir AM, Beaulieu C. Genetic and physiological determinants of *Streptomyces scabies* pathogenicity. *Mol. Plant Pathol.* 2009; 10:579–585. [PubMed: 19694949]
14. Qu Y, Spain JC. Biodegradation of 5-nitroanthranilic acid by *Bradyrhizobium* sp. strain JS329. *Appl. Environ. Microbiol.* 2010; 76:1417–1422. [PubMed: 20081004]
15. Prashar P, Kapoor N, Sachdeva S. Rhizosphere: its structure, bacterial diversity and significance. *Rev. Environ. Sci. Biotechnol.* 2013; 13:63–77.
16. Qu Y, Spain JC. Molecular and biochemical characterization of the 5-nitroanthranilic acid degradation pathway in *Bradyrhizobium* sp. strain JS329. *J. Bacteriol.* 2011; 193:3057–3063. [PubMed: 21498645]
17. Conticello SG, Langlois MA, Neuberger MS. Insights into DNA deaminases. *Nat. Struct. Mol. Biol.* 2007; 14:7–9. [PubMed: 17203067]
18. Boyen A, et al. Acetylornithine deacetylase, succinyl-diaminopimelate desuccinylase and carboxypeptidase-G2 are evolutionarily related. *Gene.* 1992; 116:1–6. [PubMed: 1628835]
19. Makarova KS, Grishin NV. The Zn-peptidase superfamily: Functional convergence after evolutionary divergence. *J. Mol. Biol.* 1999; 292:11–17. [PubMed: 10493853]
20. Rowsell S, et al. Crystal structure of carboxypeptidase G(2), a bacterial enzyme with applications in cancer therapy. *Structure.* 1997; 5:337–347. [PubMed: 9083113]
21. Shi DS, Yu XL, Roth L, Tuchman M, Allewell NM. Structure of a novel N-acetyl-L-citrulline deacetylase from *Xanthomonas campestris*. *Biophys. Chem.* 2007; 126:86–93. [PubMed: 16750290]
22. Nocek BP, Gillner DM, Fan Y, Holz RC, Joachimiak A. Structural basis for catalysis by the mono- and dimetalated forms of the dapE-encoded N-succinyl-L,L-diaminopimelic acid desuccinylase. *J. Mol. Biol.* 2010; 397:617–626. [PubMed: 20138056]
23. Martinez-Rodriguez S, et al. Mutational and structural analysis of L-N-carbamoylase reveals new insights into a peptidase M20/M25/M40 family member. *J. Bacteriol.* 2012; 194:5759–5768. [PubMed: 22904279]
24. Maret W, Li Y. Coordination dynamics of zinc in proteins. *Chem. Rev.* 2009; 109:4682–4707. [PubMed: 19728700]
25. Bar-Even A, et al. The moderately efficient enzyme: evolutionary and physicochemical trends shaping enzyme parameters. *Biochemistry.* 2011; 50:4402–4410. [PubMed: 21506553]
26. Michaelis L, Menten ML, Johnson KA, Goody RS. The original Michaelis constant: translation of the 1913 Michaelis-Menten paper. *Biochemistry.* 2011; 50:8264–8269. [PubMed: 21888353]
27. Jefferson JR, Hunt JB, Ginsburg A. Characterization of Indo-1 and Quin-2 as spectroscopic probes for Zn²⁺-protein interactions. *Anal. Biochem.* 1990; 187:328–336. [PubMed: 2116741]
28. Foster AW, Osman D, Robinson NJ. Metal preferences and metallation. *J. Biol. Chem.* 2014; 289:28095–28103. [PubMed: 25160626]
29. Wang D, Hurst TK, Thompson RB, Fierke CA. Genetically encoded ratiometric biosensors to measure intracellular exchangeable zinc in *Escherichia coli*. *J. Biomed. Opt.* 2011; 16:087011. [PubMed: 21895338]

30. Pelmentschikov V, Blomberg MRA, Siegbahn PE. A theoretical study of the mechanism for peptide hydrolysis by thermolysin. *J. Biol. Inorg. Chem.* 2002; 7:284–298. [PubMed: 11935352]
31. Slobodkin A, Reysenbach AL, Mayer F, Wiegel J. Isolation and characterization of the homoacetogenic thermophilic bacterium *Moorella glycerini* sp. nov. *Int. J. Syst. Bacteriol.* 1997; 47:969–974. [PubMed: 9336894]
32. Silverman, RB. The organic chemistry of enzyme-catalyzed reactions Ch. Vol. 6. Academic Press; 2002. p. 251–288.
33. Feig, AL.; Uhlenbeck, OC. The RNA world : the nature of modern RNA suggests a prebiotic RNA. Gesteland, Raymond F.; Cech, Thomas; Atkins, John F., editors. Vol. Ch. 12. Cold Spring Harbor Laboratory Press; 1999. p. 287–319.
34. Adams JA. Kinetic and catalytic mechanisms of protein kinases. *Chem. Rev.* 2001; 101:2271–2290. [PubMed: 11749373]
35. Steitz TA. DNA polymerases: structural diversity and common mechanisms. *J. Biol. Chem.* 1999; 274:17395–17398. [PubMed: 10364165]
36. Armstrong RN, Kondo H, Granot J, Kaiser ET, Mildvan AS. Magnetic resonance and kinetic studies of the manganese(II) ion and substrate complexes of the catalytic subunit of adenosine 3', 5'-monophosphate dependent protein kinase from bovine heart. *Biochemistry.* 1979; 18:1230–1238. [PubMed: 218617]
37. Culotta VC, Daly MJ. Manganese complexes: diverse metabolic routes to oxidative stress resistance in prokaryotes and yeast. *Antioxid. Redox Signal.* 2013; 19:933–944. [PubMed: 23249283]
38. Lambert DH, Powelson ML, Stevenson WR. Nutritional interactions influencing diseases of potato. *Am. J. Potato Res.* 2005; 82:309–319.
39. Cvetkovic A, et al. Microbial metalloproteomes are largely uncharacterized. *Nature.* 2010; 466:779–782. [PubMed: 20639861]
40. Gerlt JA, Babbitt PC. Divergent evolution of enzymatic function: mechanistically diverse superfamilies and functionally distinct suprafamilies. *Annu. Rev. Biochem.* 2001; 70:209–246. [PubMed: 11395407]

Methods References

41. Orwig SD, Lieberman RL. Biophysical characterization of the olfactomedin domain of myocilin, an extracellular matrix protein implicated in inherited forms of glaucoma. *PLoS One.* 2011; 6:e16347. [PubMed: 21283635]
42. Otwinowski Z, Minor W. Processing of X-ray diffraction data collected in oscillation mode. *Method Enzymol.* 1997; 276:307–326.
43. Terwilliger TC, et al. Decision-making in structure solution using Bayesian estimates of map quality: the PHENIX AutoSol wizard. *Acta Crystallogr. D.* 2009; 65:582–601. [PubMed: 19465773]
44. McCoy AJ, et al. Phaser crystallographic software. *J. Appl. Crystallogr.* 2007; 40:658–674. [PubMed: 19461840]
45. Emsley P, Lohkamp B, Scott WG, Cowtan K. Features and development of Coot. *Acta Crystallogr. D.* 2010; 66:486–501. [PubMed: 20383002]
46. Adams PD, et al. PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr. D.* 2010; 66:213–221. [PubMed: 20124702]
47. Moriarty NW, Grosse-Kunstleve RW, Adams PD. Electronic Ligand Builder and Optimization Workbench (eLBOW): a tool for ligand coordinate and restraint generation. *Acta Crystallogr. D.* 2009; 65:1074–1080. [PubMed: 19770504]
48. Konarev PV, Volkov VV, Sokolova AV, Koch MHJ, Svergun DI. PRIMUS: a Windows PC-based system for small-angle scattering data analysis. *J. Appl. Crystallogr.* 2003; 36:1277–1282.
49. Svergun DI. Restoring low resolution structure of biological macromolecules from solution scattering using simulated annealing. *Biophys. J.* 1999; 76:2879–2886. [PubMed: 10354416]
50. Volkov VV, Svergun DI. Uniqueness of ab initio shape determination in small-angle scattering. *J. Appl. Crystallogr.* 2003; 36:860–864.

51. Kozin MB, Svergun DI. Automated matching of high- and low-resolution structural models. *J. Appl. Crystallogr.* 2001; 34:33–41.
52. McWilliam H, et al. Analysis Tool Web Services from the EMBL-EBI. *Nucleic Acids Res.* 2013; 41:W597–600. [PubMed: 23671338]
53. Tung CH, Huang JW, Yang JM. Kappa-alpha plot derived structural alphabet and BLOSUM-like substitution matrix for rapid search of protein structure database. *Genome Biol.* 2007; 8:16.
54. Krissinel E, Henrick K. Inference of macromolecular assemblies from crystalline state. *J. Mol. Biol.* 2007; 372:774–797. [PubMed: 17681537]
55. Chovancova E, et al. CAVER 3.0: a tool for the analysis of transport pathways in dynamic protein structures. *PLoS Comput. Biol.* 2012; 8:e1002708. [PubMed: 23093919]
56. Schwede T, Kopp J, Guex N, Peitsch MC. SWISS-MODEL: An automated protein homology-modeling server. *Nucleic Acids Res.* 2003; 31:3381–3385. [PubMed: 12824332]
57. Kozakov D, et al. The FTMap family of web servers for determining and characterizing ligand-binding hot spots of proteins. *Nat. Protoc.* 2015; 10:733–755. [PubMed: 25855957]
58. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol. Biol. Evol.* 2013; 30:2725–2729. [PubMed: 24132122]
59. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* 2004; 32:1792–1797. [PubMed: 15034147]
60. Jones DT, Taylor WR, Thornton JM. The rapid generation of mutation data matrices from protein sequences. *Comput. Appl. Biosci.* 1992; 8:275–282. [PubMed: 1633570]

Figure 1. Overall structure and substrate binding features of 5NAA-A

a, Octameric crystal structure of 5NAA-A, colored by protomer (apo structure shown).

Overall dimensions, dimerization, and tetramerization interfaces are labeled. **b,** Dimer unit labeled with location of two active sites. **c,** Substrate binding details. Top: Superposition of apo and 5NAA-bound structures in binding pocket. Loop1 (residues 91–108) and Loop 2 (residues 393–397) are disordered in the apo structure (red/dark blue) but adopt an ordered conformation when 5NAA is bound (cyan/grey). Bottom: 5NAA binding interactions with protein residues, superimposed with apo structure.

Figure 2. Dependence of 5NAA-A on metal ions for activity

a, Effect of divalent transition or alkaline earth metals on 5NAA-A activity as measured by 5NAA depletion (500 μM metal ion, 0.25 μM 5NAA-A). **b**, Concentration dependence of Mn^{2+} and Zn^{2+} . For each metal and at each concentration the reaction was run in triplicate. Data are presented as mean $\pm$ standard error of the mean (SEM). **c**, Kinetics analysis for 5NAA-A in the presence of first row transition metal ions and representative mutant 5NAA- A_{R289A} . For Mn^{2+} and Zn^{2+} analyses, three independent enzyme samples were tested with each substrate concentration measured in triplicate. For Co^{2+} , Cu^{2+} with wild-type 5NAA-A, and Mn^{2+} , Zn^{2+} with 5NAA- A_{R289A} , two independent enzyme samples were tested in triplicate. For Fe^{2+} , one enzyme preparation was tested anaerobically, in triplicate. Data are reported as mean $\pm$ SEM.

Figure 3. Metal ion, substrate, and product binding to 5NAA-A measured by ITC

Representative raw data and plots of integrated heat versus molar ratio with calculated K_d values where appropriate. **a**, Mn^{2+} only as titrant. **b**, 5NAA or 5NSA only. **c**, 5NSA and Mn^{2+} titrated together in 1:1 mixture. For (**a–c**), 150 μM of titrant indicated was injected in 9 μl aliquots to 6.4 μM enzyme. Titrations were conducted three times and calculated parameters are mean $\pm$ standard deviation. **d**, 5NAA-A was pre-incubated with 150 μM 5NAA at room temperature for 30 min prior to injection of 150 μM Mn^{2+} aliquots. The experiment was performed twice. See also Supplementary Fig. 5b,c.

a, Superposition of metal binding residues of apo 5NAA-A (red), 5NAA-bound (cyan), Mn^{2+} /5NSA-bound (magenta) 5NAA-A and Zn^{2+} /Tet-bound 5NAA-A_{R289A} (yellow). **b**, Coordination environment and representative electron density for Mn^{2+} /5NSA-bound 5NAA-A. **c**, Coordination environment and representative electron density for Zn^{2+} and Tet in the active site of Zn^{2+} /Tet-bound 5NAA-A_{R289A} structure in two orientations, highlighting stabilization of the intermediate by Glu₁₅₈. 2Fo – Fc electron density (blue) is contoured at 2.0σ and difference Fo – Fc density (green) is contoured at 2.5σ . The Fo – Fc density map was calculated using coordinates of the refined model prior to addition of 5NSA or Tet. **d**, Proposed nucleophilic aromatic substitution mechanism for hydrolysis of 5NAA to 5NSA by 5NAA-A. M^{2+} , activating metal ion. Grey molecules/wavy bonds: inferred based on available structures.