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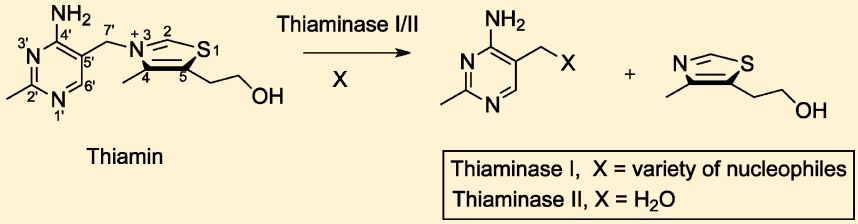
Structure of a *Clostridium botulinum* C143S Thiaminase I/Thiamin Complex Reveals Active Site Architecture[†,‡,‖,‡]

Megan D. Sikowitz,[§] Brateen Shome,[∥] Yang Zhang,[§] Tadhg P. Begley,*[,∥] and Steven E. Ealick*[,§]

[§]Department of Chemistry and Chemical Biology, Cornell University, Ithaca, New York 14853, United States

[∥]Department of Chemistry, Texas A&M University, College Station, Texas 77843, United States

Supporting Information



ABSTRACT: Thiaminases are responsible for the degradation of thiamin and its metabolites. Two classes of thiaminases have been identified based on their three-dimensional structures and their requirements for a nucleophilic second substrate. Although the reactions of several thiaminases have been characterized, the physiological role of thiamin degradation is not fully understood. We have determined the three-dimensional X-ray structure of an inactive C143S mutant of *Clostridium botulinum* (Cb) thiaminase I with bound thiamin at 2.2 Å resolution. The C143S/thiamin complex provides atomic level details of the orientation of thiamin upon binding to Cb-thiaminase I and the identity of active site residues involved in substrate binding and catalysis. The specific roles of active site residues were probed by using site directed mutagenesis and kinetic analyses, leading to a detailed mechanism for Cb-thiaminase I. The structure of Cb-thiaminase I is also compared to the functionally similar but structurally distinct thiaminase II.

Thiamin (vitamin B_1) is an essential vitamin in all living organisms. Thiamin diphosphate (ThDP) is a cofactor in many biological processes including carbohydrate metabolism and amino acid biosynthesis. Most plants, fungi, and bacteria can synthesize thiamin, but animals must obtain it from their diets. Numerous investigations over the past 60 years provide a nearly complete understanding of thiamin biosynthesis,[1] yet the physiological basis for its degradation and the fate of the breakdown products are largely unknown. Thiaminases catalyze the degradation of thiamin into its thiazole and pyrimidine components (Figure 1).[2−10] These enzymes are found in a wide variety of organisms, including plants, fish, and bacteria. Consequently, ingestion of thiaminase containing foods by humans or other animals can cause symptoms of thiamin deficiency.[11,12] In humans, this deficiency can affect the cardiovascular system (wet beriberi) or nervous system (dry beriberi) with potentially fatal consequences.[13,14]

Biochemical analyses have established two distinct classes of thiaminases. Thiaminase I utilizes a variety of nucleophiles,[15,16] whereas thiaminase II exclusively utilizes water as the nucleophile.[17] Thiaminase II[17] is distinct from thiaminase I in both structure and sequence.[18] A crystal structure of the thiaminase I from *Bacillus thiaminolyticus* (Bt-thiaminase I) has been previously reported.[18] Bt-thiaminase I is a monomer composed of two domains joined by three crossover segments and is structurally homologous to the group II periplasmic binding proteins (PBPs).[18,19] PBPs bind small molecules and deliver them to ABC transporters for eventual uptake into the cytoplasm. Structural studies reveal group II PBPs in an open conformation when no ligand is bound and in a closed conformation when a ligand is bound.[19] Bt-thiaminase I is a rare example of an enzyme in the group II PBP superfamily. Bt-thiaminase I is structurally similar to TbpA,[20] which is the PBP in many prokaryotes for thiamin, thiamin phosphate (ThMP), and thiamin diphosphate (ThDP). Interestingly, THI5, the thiamin pyrimidine synthase in eukaryotes, is also a member of the group II PBP superfamily.[21,22] THI5 is structurally homologous to ThiY,[21] which is the PBP in some prokaryotes for the thiamin degradation product *N*-formyl-4-amino-5-(aminomethyl)-2-methylpyrimidine.[23]

Previous biochemical and structural studies established that an active site cysteine residue is involved in thiaminase I catalysis.[15,16,18] A crystal structure of Bt-thiaminase I with the mechanism-based, irreversible inhibitor 4-amino-6-chloro-2,5-dimethylpyrimidine (ACDP) showed that the active site is located in a V-shaped cleft created between the two Bt-thiaminase I α/β domains.[18] The active site location is structurally similar to the ligand binding site in the PBPs. The active site cysteine residue is activated by a nearby glutamate residue for addition to the thiamin pyrimidine at C6′.

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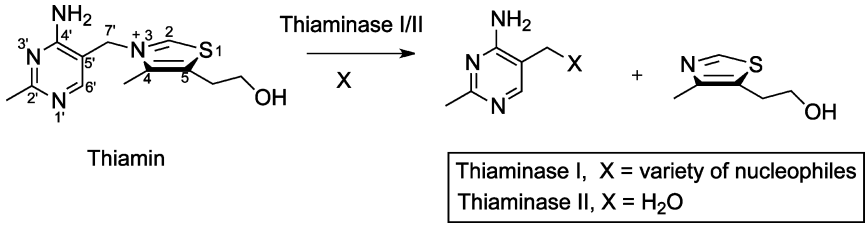

Figure 1. Thiamin cleavage reaction catalyzed by thiaminases. Thiaminase I utilizes a variety of nucleophiles, whereas thiaminase II exclusively uses water.

Loss of thiazole followed by reversal of these steps, using a variety of nucleophiles, completes the thiaminase I-catalyzed reaction. Despite their highly distinct sequences and structures, thiaminase I and most thiaminase IIs utilize an activated cysteine residue to form a covalently bound intermediate.[17,18]

Thiaminase I from *Clostridium botulinum* (Cb-thaminase I) is a 46.3 kDa protein with 51% sequence identity to Bt-thiaminase I. The catalytic cysteine residue (Cys135) was predicted by sequence alignment with other thiaminase Is and was mutated to generate a catalytically inactive Cb-thiaminase I (C143S). C143S was cocrystallized with thiamin to obtain the C143S/thiamin complex reported here. This is the first structure of a thiaminase I/substrate complex and it reveals atomic level details of thiamin binding prior to degradation. This structure was used to identify the key amino acids involved in the Cb-thiaminase I reaction. Site directed mutagenesis and kinetic studies of thiamin degradation were used to assign roles to active site amino acid residues. Together, the C143S/thiamin structure and subsequent kinetic studies led to a detailed mechanistic proposal for thiaminase I. The active sites and catalytic mechanisms of Cb-thiaminase I and thiaminase II are also compared.

MATERIALS AND METHODS

Cloning, Overexpression, and Purification of Cb-Thiaminase I. The thiaminase I gene, *bcmE*, was cloned from *C. botulinum* A str. ATCC 19397 genomic DNA. Standard DNA manipulation methods were used for all of the cloning procedures. The gene was inserted into pTHT, a modified pET-28 plasmid with an N-terminal His_6 tag, followed by a tobacco etch virus (TEV) protease cleavage site. All active site mutants were obtained by site-directed mutagenesis of the native gene using standard, PCR-based mutagenesis.[24]

After DNA sequencing to verify plasmid accuracy, we transformed the plasmids into *Escherichia coli* BL21(DE3) cells and the cells were grown overnight at 37 °C on selective kanamycin (30 μg/mL) containing agar. Starter cultures were then grown from a single colony. A selected colony was placed in 10 mL of sterile Luria-Bertani (LB) media containing 30 μg/mL kanamycin at 37 °C with shaking overnight. The following day, 5 mL of the overnight starter culture was added directly to 1.5 L volumes of sterile LB media. Cells grew with shaking at 37 °C until reaching an OD_{600} of 0.6, at which point the incubator temperature was reduced to 15 °C. Once an induction temperature of 15 °C and an OD_{600} of 0.8 were reached, protein overexpression was initiated by adding isopropyl β-D-1-thiogalactopyranoside to the cultures with a final concentration of 0.5 mM. Cells were harvested 18 h after induction of protein overexpression by centrifugation at 2000*g* for 20 min. The cell pellet was collected and frozen at −20 °C for storage.

The cell pellet was later thawed and resuspended in 45 mL of lysis buffer (50 mM Tris−HCl, 300 mM NaCl, and 20 mM imidazole at pH 8.0) before lysing by sonication. The lysed cell extract was centrifuged at 40,000*g* for 30 min at 4 °C. The supernatant was collected and loaded onto a 2 mL Ni-nitrilo acetic acid (NTA) column (Qiagen), pre-equilibrated with lysis buffer. The column was then washed with 45 mL of lysis buffer to remove any nonspecifically bound contaminants from the column. The protein was eluted from the column by passing 20 mL of elution buffer (50 mM Tris−HCl, 300 mM NaCl, and 250 mM imidazole at a final pH of 8.0) through the column. The elution volume containing Cb-thiaminase I was collected. This procedure was followed for purification of Cb-thiaminase I and all active site mutant proteins. In addition to Ser143, Tyr46, Tyr80, Asp94, Glu271, and Asp302 were selected for mutation on the basis that the side chain interacted directly with the substrate or is potentially involved in activating residues that interact with the substrate.

To remove the His_6 tag for crystallization, the purified C143S Cb-thiaminase I was incubated with TEV protease during dialysis into 3.5 L of 10 mM Tris HCl at pH 8.0, 150 mM KCl and 1 mM dithiothreitol for 18 h at 4 °C, utilizing a ratio of 0.5 mg of TEV for every 10 mg of C143S. The sample was then passed over a Ni-NTA column preequilibrated with lysis buffer. The elution volume containing C143S with the His_6 tag removed was collected in the flow through. Complete cleavage and purity were assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The sample was buffer exchanged by overnight dialysis into 10 mM Tris−HCl at pH 8.0 and 150 mM KCl, then concentrated to 15 mg/mL using a centrifugal filter device with a molecular weight cutoff of 10 000 Da (Vivaspin). The purification process yielded approximately 3.5 mg protein per L of cell culture. The purified protein was aliquoted and flash frozen in liquid nitrogen for storage at −80 °C.

Cocrystallization of C143S and Thiamin. For crystallization using the hanging drop vapor diffusion method, the protein was diluted with a solution of 10 mM Tris pH 8.0, 150 mM KCl and 10 mM thiamin with final concentrations of 7.5 mg/mL protein and 5 mM thiamin. Equal volumes of protein and reservoir solutions were mixed and equilibrated at 18 °C against a total volume of 500 μL well solution. The initial crystallization condition was determined using the commercially available Wizard III sparse matrix screen (Emerald Biosystems). The optimized crystallization condition was 22% (w/v) polyethylene glycol 10 000, 100 mM sodium citrate pH 4.4, and 2% (v/v) dioxane. Needlelike C143S/thiamin crystals grew approximately 200 μm long and 10−20 μm thick in 3−5 days. Crystals were cryoprotected for data collection in a solution composed of the mother liquor supplemented with 20% (v/v) ethylene glycol.

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X-ray Data Collection and Processing. X-ray diffraction data were measured at the Northeastern Collaborative Access Team (NE-CAT) beamline 24-ID-C, at the Advanced Photon Source, Argonne National Laboratory, using a Quantum 315 detector (Area Detector Systems Corp.) at a distance of 200 mm. The oscillation method was used with 1.0° rotation per frame for 180° with cryocooling. Diffraction data were measured at a wavelength of 0.979 18 Å. The NE-CAT in-house automated RAPD data collection and processing system, which utilizes XDS,[25,26] was used for indexing, integration, and scaling of the data. Data collection and processing statistics are listed in Table 1.

Structure Determination, Model Building, and Refinement. The structure of the C143S/thiamin complex was determined by the molecular replacement method using thiaminase I from *B. thiaminolyticus* (PDB ID: 2THI), with a sequence identity of 51%, as the search model. The crystal belongs to space group $P2_1$ and contains two molecules per asymmetric unit corresponding to a Matthews coefficient[27] of 1.96 Å^3/Da and estimated solvent content of 37%. MolRep[28] positioned two chains in the asymmetric unit. Iterative rounds of model refinement were conducted using COOT[29] for manual model building followed by refinement with PHENIX.refine[30] using default parameters. The thiamin substrate was added using PHENIX.ligandfit for placement into $F_o - F_c$ electron density. Water molecules were added during later rounds of refinement. The quality of the structure was analyzed using MolProbity[31] and COOT.[29] Structure refinement converged with a final *R*-factor of 15.9% and R_{free} of 21.1%. Complete refinement statistics are listed in Table 1.

Table 1. Summary of Data Collection and Refinement Statistics

	C143S/Thiamin
beamline	APS 24-ID-C
resolution (Å)	2.18
wavelength (Å)	0.97918
space group	$P2_1$
a (Å)	63.5
b (Å)	36.3
c (Å)	156.8
β (deg)	91.4
Matthews coefficient	1.96
% solvent	37.4
mol/asu	2
measured reflections	132181
unique reflections	37678
average I/σ	16.3 (5.0)[a]
redundancy	3.4 (3.0)
completeness (%)	99 (92)
R_{sym} (%)[b]	6.5 (29.4)
no. of protein atoms	5814
no. of ligand atoms	66
no. of water atoms	430
reflections in working set	37536
reflections in test set	1877
R-factor/R_{free} (%)[c]	15.9/21.1
rms deviation from ideals	
bonds (Å)	0.008
angles (deg)	1.045
average *B* factor for protein (Å^2)	21.7
average *B* factor for water (Å^2)	26.3
average *B* factor for ligand (Å^2)	26.3
Ramachandram plot	
most favored (%)	99.0
allowed (%)	1.0
disallowed (%)	0.0

[a]Values in parentheses are for the highest resolution shell. [b]$R_{merge} = \Sigma\Sigma_i|I_i - \langle I\rangle|/\Sigma\langle I\rangle$, where $\langle I \rangle$ is the mean intensity of the *N* reflections with intensities I_i and common indices *h*,*k*, and *l*. [c]*R*-factor = $\Sigma_{hkl}||F_{obs}| - k|F_{cal}||/\Sigma_{hkl}|F_{obs}|$, where F_{obs} and F_{cal} are observed and calculated structure factors, respectively, calculated over all reflections used in the refinement. R_{free}, is similar to R_{work} but calculated over a subset of reflections (5%) excluded from all stages of refinement.

Determination of Kinetic Parameters. Steady state kinetic analysis was performed for six Cb-thiaminase I mutant proteins that were selected on the basis of the crystal structure of the C143S/thiamin complex. Reactions were carried out in 100 mM potassium chloride and 50 mM phosphate buffer at pH 8.0 using 713 mM *β*-mercaptoethanol as the exogenous nucleophile. All reactions were incubated at 25 °C except the E271Q Cb-thiaminase I, which was measured at 37 °C due to low activity levels. The reactions were initialized by the addition of enzyme to the reaction volume containing thiamin, with thiamin concentrations ranging from 100 *μ*M to 10 mM. Aliquots were taken from the reaction mixture after defined time intervals, quenched with 1 M HCl, filtered through a 10 000 Da cutoff membrane to remove the enzyme and analyzed by HPLC.

In the HPLC method, the ratio of 100 mM phosphate buffer at pH 6.6 (P) to methanol (M)/water mixture was varied over time in minutes (*t*). The gradient was carried out following the scheme (*t*, M%, H_2O%, P%): (0, 0, 0, 100), (5, 0, 10, 90), (9, 15, 25, 60), (14, 65, 20, 15), (19, 0, 0, 100), (25, 0, 0, 100). Peak areas of the product in the enzyme assays were measured and the concentrations were calculated based on a calibration curve relating peak area to 4-methyl-5-hydroxyethylthiazole concentration. The initial rates were calculated from plots of concentration versus time. Initial rates were then plotted and fit to the Michaelis–Menten equation for calculation of kinetic parameters.

Figure Preparation. All figures were prepared using PyMOL[32] or ChemDraw (Cambridge Biosoft) and compiled in Photoshop (Adobe).

RESULTS

Overall Structure of Cb-Thiaminase I. The structure of the C143S/thiamin complex, with two molecules per asymmetric unit, was determined at 2.2 Å resolution. Of the 404 possible amino acid residues in the Cb-thiaminase I sequence, the structure contains residues 39–404 for molecule A and 40–404 for molecule B. Each molecule contains thiamin, citrate, and two metal ions modeled as Mg. The two molecules (A and B) make a total of 16 nonbonded interactions along an interface area of approximately 210 Å^2, suggesting only crystal packing contacts. Size exclusion chromatography (data not shown) predicted that Cb-thiaminase I is a monomer in solution. This is also consistent with the oligomeric state of Bt-thiaminase I.[18]

Cb-thiaminase I is a member of the group II PBP superfamily and can be divided into two distinct domains (Figure 2). The N-terminal domain is composed of residues 39–120 and 297–384, whereas residues 145–296 and 385–403 form the C-terminal domain. Both domains have a central *β*-sheet flanked on either side by *α*-helices with three crossover segments

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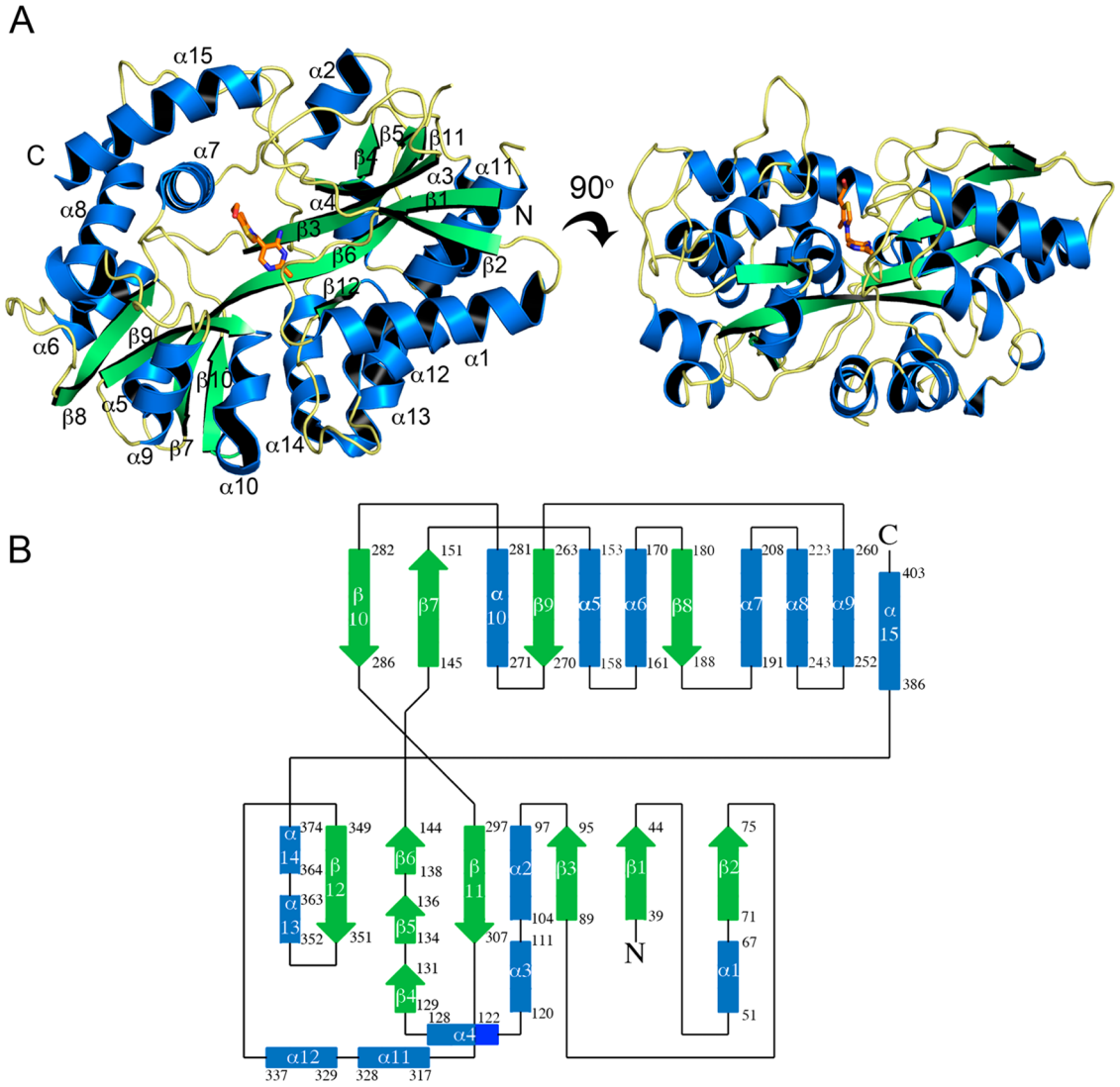

Figure 2. Overall structure of C143S/thiamin. (A) The ribbon diagram shows α/β fold of C143S with α-helices depicted in blue and β-strands in green. The two domains are composed of β-sheets flanked by α-helices on both sides, connected by a central β-strand. The thiamin-binding site is located in the central groove between the N- and C-terminal domains. The 90° rotation shows the orientation of the thiamin molecule in the binding cleft. (B) The topology diagram of C143S following the same coloring scheme as in (A).

connecting the N- and C-terminal domains. Residues 144−145, 287−296, and 375−385 comprise the three domain linker regions. The crossover segment formed by residues 287−296 connects β_{10} to β_{11}, and the crossover segment formed by residues 375−384 connects α_{14} and α_{15}. The third crossover connects β_6 to β_7 at residue 144.

Cb-Thiaminase I Active Site and Substrate Binding. The structure of the C143S/thiamin complex reveals the substrate binding site and atomic level details of binding interactions (Figure 3). Each monomer contains one thiamin molecule in a V-shaped cleft between the two domains. This cleft is approximately 17 Å deep, 15 Å long, and 12 Å wide. Six tyrosine residues, Tyr46, Tyr48, Tyr80, Tyr252, Tyr269, and Tyr300, form an outer collar of the cleft with most of the active site residues located at the bottom of the cleft. The catalytic cysteine residue Cys143, which is represented by Ser143 in the C143S/thiamin complex, is located on β_6.

Thiamin binds to the active site in the F-conformation with torsion angles of ϕ_T = −10° (C5′−C7′−N3−C2) and ϕ_P = −93.6° (N3−C7′−C5′−C4′).[33] The N3−C7′−C5′angle is

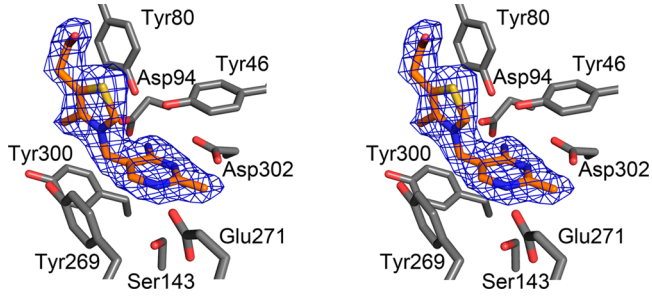

Figure 3. C143S/thiamin active site interactions . The stereodiagram displays active site residues near the thiamin binding site with thiamin modeled into a $F_o - F_c$ electron density map contoured at 1σ.

108°. The thiamin pyrimidine and thiazolium moieties are approximately perpendicular with a dihedral angle of 85.9° between their two planes. The pyrimidine portion of the thiamin is positioned toward the bottom of the V-shaped binding pocket (Figure 4). The thiamin N3′ atom is positioned 2.8 Å from the Asp302 carboxylic acid group, within hydrogen

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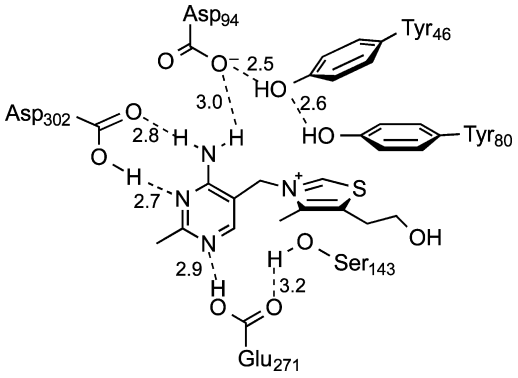

Figure 4. Thiamin binding interactions in C143S thiaminase I. All distance values given are in Å.

bond distance if Asp302 is protonated. The 4′-amino group hydrogen bonds with Asp94 (2.9 Å), which in turn hydrogen bonds to the Tyr46 hydroxyl group (2.5 Å). Tyr80 is 3.5 Å from the positivity charged N3 atom, and hydrogen bonds to the Tyr46 hydroxyl group (2.9 Å). The thiazolium group does not interact with the enzyme except through van der Waals contacts and is extended toward the top of the binding groove with the 5-hydroxyethyl group extended out toward the solvent region.

Steady State Kinetics of Cb-Thiaminase I and Mutant Proteins. Thiamin degradation by Cb-thiaminase I was determined by using high-performance liquid chromatography (HPLC) to detect the reaction products. Wild-type Cb-thiaminase I catalyzes the thiaminase reaction with a $k_{cat.}$ of 2.31×10^2 s^{-1} and a $k_{cat.}/K_m$ value of 5.10×10^5 M^{-1} s^{-1}. All activity was abolished by mutation of the catalytic cysteine in C143S. The kinetic parameters for five other Cb-thiaminase I active site mutants are reported in Table 2. A description of the

Table 2. Kinetic Properties of Thiaminase Activity for Wild-Type Cb-Thiaminase I and Active Site Mutants

mutant	$k_{cat.}$ (s^{-1})	K_M (M)	$k_{cat.}/K_M$ (M^{-1} s^{-1})
wild type	$(2.31 \pm 1.10) \times 10^2$	$(4.52 \pm 0.51) \times 10^{-4}$	5.10×10^5
C143S	nd[a]	nd[a]	
Y80F	1.07 ± 0.12	$(2.11 \pm 0.81) \times 10^{-3}$	4.96×10^2
D302N	8.14 ± 0.74	$(3.07 \pm 1.09) \times 10^{-5}$	2.64×10^4
E271Q	$(1.1 \pm 0.03) \times 10^{-2}$	$(7.47 \pm 1.25) \times 10^{-5}$	1.50×10^2
Y46F	$6.09 \pm 0.38 \times 10^1$	$(2.45 \pm 0.51) \times 10^{-4}$	2.52×10^5
D94N	$(2.3 \pm 0.2) \times 10^{-1}$	$(3.49 \pm 0.76) \times 10^{-3}$	6.61×10^1

[a](nd) Activity not detected at the level of sensitivity of the assay.

determination of the pH rate profile for the native enzyme is provided in the Supporting Information and the profile is shown in Figure S2 (Supporting Information).

DISCUSSION

Structural Comparison of the Cb-Thiaminase I with Other Proteins. A DALI[34] search was performed to identify proteins with high structural similarity to Cb-thiaminase I (Table 3). Cb-thiaminase I showed the highest structural homology to Bt-thiaminase I (PDB ID: 2THI)[18] with a root mean squared deviation (RMSD) of 0.9 Å. Bt-thiaminase I, which was used as the search model for molecular replacement, has 51% sequence identity with Cb-thiaminase I. Cb-thiaminase I is a member of the group II PBP superfamily, with three

Table 3. Enzymes Structurally Similar to Cb-Thiaminase I

protein	PDB ID	Z score	rmsd	% identical	no. of aligned residues
thiaminase I	2THI	57.2	0.9	51	361
maltose PBP	1EU8	28.6	3.3	17	328
β-D-galctopyranose PBP	3OO6	26.2	3.4	11	390
cyclodextrin PBP	2DFZ	25.2	3.4	16	323
iron PBP	1O7T	21.2	3.0	10	287
thiamin PBP	2QRY	18.3	3.9	14	308

characteristic crossover segments. Most superfamily members bind small molecules including metals, vitamins, sugars, or amino acids and are components of ABC transporters.[35] The small molecule binding site is located in a cleft near the surface that corresponds structurally to the Cb-thiaminase I active site. The DALI search identified several PBPs with RMSD values in the range 3.0−3.5 Å. The thiamin binding protein, TbpA, shows an RMSD of 3.9 Å. Thiaminase I is a rare example of a PBP superfamily member with enzymatic activity. The structural similarity between thiaminase I and TbpA suggests an evolutionary relationship between these two PBP group II superfamily members.[20]

PBP proteins undergo a conformational change when the small molecule ligand binds. The two domains are in an open state when no ligand is bound and close like a Venus fly trap after ligand binding.[36] A comparison between the structures of the C143S/thiamin complex and unliganded Bt-thiaminase I (2THI)[18] suggests that thiaminase I does not undergo a conformational change upon binding thiamin (Figure 5A). Both the liganded and unliganded forms appear to most closely resemble the closed conformation of group II PBPs.

Comparison of Cb-Thiaminase I and Bt-Thiaminase I. The previous structure of Bt-thiaminase I with the mechanism-based inhibitor 4-amino-6-chloro-2,5-dimethylpyridimine (ACDP), which lacks the thiazolium moiety, identified the catalytic cysteine residue and the general location of the active site.[18] Attempts to model the thiamin binding geometry by using the inactivated thiaminase I structure were unreliable because the covalent bond between C6 of the inhibitor and Sγ of the cysteine side chain, followed by rearomatization of the pyrimidine ring, distorted the active site interactions. Therefore, the structure of thiaminase I with the suicide inhibitor ACDP does not represent any possible intermediate along the reaction pathway. Consequentially, the structure of the Bt-thiaminase I-ACDP adduct showed few active site interactions and only one hydrogen bond. In contrast, the C143S/thiamin complex reveals all interactions within the enzyme/substrate complex. The thiamin pyrimidine ring in the C143S/thiamin complex forms four hydrogen bonds with active site residues (Figure 4). These include interactions between the 4′-amino group of the thiamin pyrimidine and the side chain of Asp94. Additionally, Asp302 hydrogen bonds to both the 4′-amino group and N3′, and Glu271 hydrogen bonds to Glu271. Comparison of the structures of the C143S/thiamin complex and the Bt-thiaminase-ACDP adduct shows that ACDP and the thiamin pyrimidine are approximately perpendicular (Figure 5B).

The C143S/thiamin complex structure also reveals the binding geometry of the thiamin thiazolium moiety, which is pointed toward the solvent at the top of the binding cleft. In the crystal structure, no hydrogen bonds or stacking interactions with any active site residues are observed. Previous studies showed that thiaminase I accepts thiamin analogs with modified

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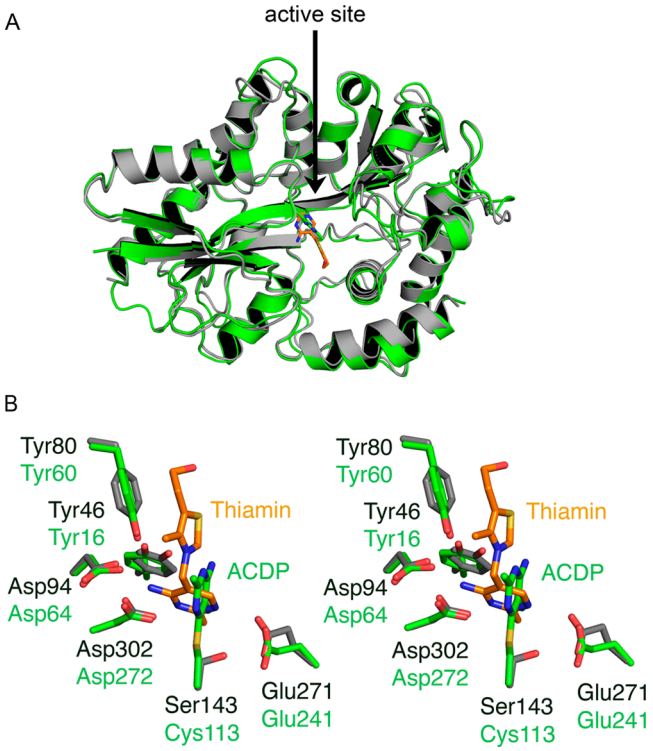

Figure 5. Comparison with thiaminase I/ACDP structure from *B. thiaminolyticus*. (A) C143S structure (gray) with thiamin (orange) superimposed on Bt-thiaminase I with covalently bound inhibitor (green, PDB ID: 4THI). (B) Active site comparison between C143S and Bt-thiaminase I following the same coloring scheme as in (A). The pyrimidine portion of thiamin from the C143S/thiamin complex is oriented perpendicular to the ACDP inhibitor covalently bound to the active site cysteine in Bt-thiaminase I.

thiazolium ring moieties; however, modifications to the pyrimidine are not tolerated.[37] These observations are consistent with the positioning of the thiamin molecule in the active site of Cb-thiaminase I.

Comparison to Thiamin Binding Sites in Other Proteins. In addition to thiaminases, biological macromolecules that bind thiamin, or its monophosphorylated or diphosphorylated forms, include ThDP-dependent enzymes, thiamin biosynthetic enzymes, thiamin transport proteins and riboswitches. Thiamin has been observed bound in three different conformations: F,V and S. The F-conformation is the lowest energy form in solution and is defined by $\phi_T \approx 0°$ and $\phi_P \approx \pm 90°$, where ϕ_T is the C5′−C7′−N3−C2 torsion angle and ϕ_P is the N3−C7′−C5′−C4′ torsion angle. The V-conformation is defined by $\phi_T \approx \pm 90°$ and $\phi_P \approx 90°$, and the rare S-conformation is defined by $\phi_T \approx \pm 100°$, $\phi_P \approx \pm 150°$. A common thiamin-binding motif is found in enzymes that utilize ThDP as a cofactor, where thiamin binds in the V-conformation.[33,38] This 30-residue motif begins with −GDG− and concludes with −NN. ThDP binding is usually facilitated by phosphate binding interactions with a divalent metal, aspartate side chains, and asparagine side chains.[39]

The thiamin, ThMP, and ThDP binding sites found in enzymes that are not ThDP-dependent show significant divergence. Thiamin pyrophosphokinase (TPK) and ThMP kinase, like Cb-thiaminase I, bind thiamin in the F-conformation; however, their thiamin binding motifs show little similarity. TPK (PDB ID: 1IG3) pyrophosphorylates thiamin to ThDP and binds thiamin ($\phi_T = -6°$, $\phi_P = -78°$) through multiple interactions.[40] In addition to stacking interactions with a nearby tryptophan residue, the thiamin pyrimidine moiety hydrogen bonds through its 4′-amino group to an aspartate side chain and a main chain carbonyl group. An additional hydrogen bond forms between N1′ and a nearby serine residue. ThDP, the TPK product, is further anchored through a phosphate-binding pocket. In ThMP kinase (PDB ID: 3C9T), ThDP ($\phi_T = -20°$, $\phi_P = -64°$) is bound in a cleft with stacking interactions between the thiazolium moiety and a nearby tryptophan residue.[41] In addition to phosphate binding pocket interactions, ThDP hydrogen bonds to a glutamate side chain through its 4′-amino group. Although all three enzymes hydrogen bond to the 4′-amino group by using either a glutamate or aspartate side chain, they share few other thiamin binding features. TPK and ThMP kinase contain phosphate-binding pockets that facilitate binding of ThMP or ThDP; however, Cb-thiaminase I binds thiamin with its hydroxyethyl substituent at C5 extended outward from the binding cleft toward the solvent. ThMP in thiamin phosphate synthase (PDB ID: 2TPS) displays a nonstandard "cis" V-conformation ($\phi_T = -95.6°$, $\phi_P = -113°$).[42] Like Cb-thiaminase I, its pyrimidine is located at the bottom of its active site. Thiamin binding is aided by hydrogen bonding between its 4′-amino group and N3′ and with a glutamine side chain within an otherwise hydrophobic pyrimidine binding pocket.

Despite sharing only 14% sequence identity, both Cb-thiaminase I and *E. coli* thiamin binding protein have a group II PBP fold and have similar binding site locations for thiamin; however, their thiamin binding motifs differ. In the reported structure of thiamin binding protein (PDB ID: 2QRY), ThMP spans the length of the binding cleft in an F-conformation ($\phi_T = 0°$, $\phi_P = -83°$).[20] Thiamin binding protein binds ThMP by anchoring the phosphate group within a hydrogen bond rich pocket and by sandwiching the thiazolium group between two tyrosine residues. The ThMP pyrimidine interacts mainly through water mediated hydrogen bonding with one hydrogen bond to a serine residue side chain. In contrast, in Cb-thiaminase I the thiamin pyrimidine is buried at the bottom of a cleft, with multiple hydrogen bonds and with the thiazolium portion extending upward toward the solvent.

Thiamin biosynthesis is often under the control of a ThMP riboswitch in mRNA. A structure for the ThMP riboswitch (PDB ID: 2HOM) shows that ThMP binds ThMP in the rare S-conformation ($\phi_T = -85°$, $\phi_P = -173°$).[43] The riboswitch forms hydrogen bonds between N3′ and the 4′-amino group of the ThMP pyrimidine and the 2-amino group and N3, respectively, of an adenine base. An additional hydrogen bond forms between ThMP N1′ and a ribosyl 3-hydroxyl group. A weak hydrogen bond is observed between the ThMP sulfur atom and a ribosyl 2-hydroxyl group. The phosphate group interacts with the O6 atoms of a pair of guanine bases, through a magnesium ion.

Cb-Thiaminase I Mechanism. A mechanistic proposal for thaminase I is shown in Figure 6. In this mechanism, Glu271 functions as the base activating Cys143 for addition to the pyrimidine as previously proposed.[15,18] This reaction is essential for activating the pyrimidine for side chain substitution: the C143S mutant is inactive and $k_{cat.}$ for the E271Q mutant is reduced 21 000-fold (Table 2). Asp302 is hydrogen bonded to N3 of the pyrimidine, suggesting that this residue also activates the pyrimidine ring for nucleophilic attack. This is supported by the observation that $k_{cat.}$ of the

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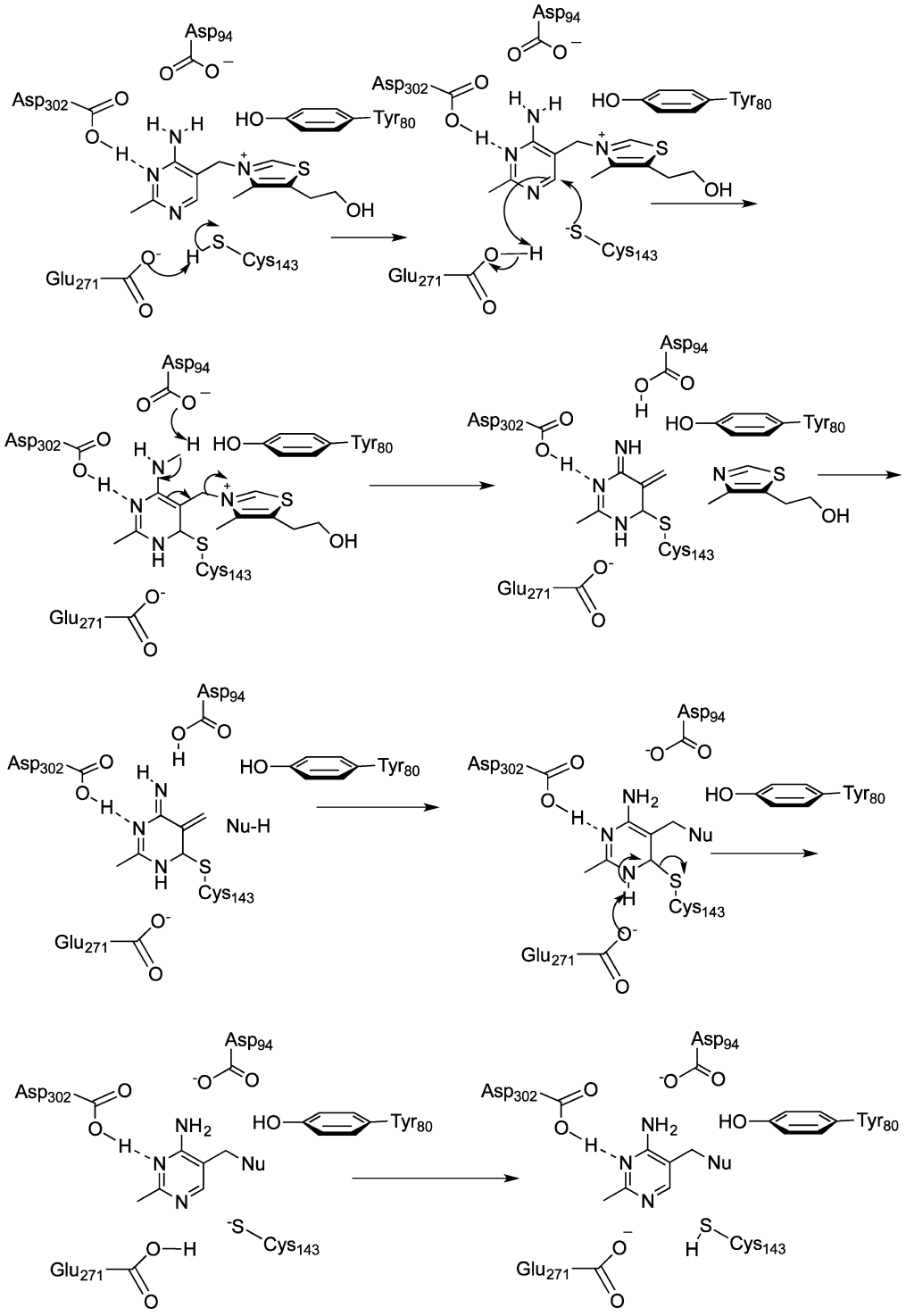

Figure 6. Mechanism of thiamin degradation by Cb-thiaminase I. This mechanism is based on kinetic studies of Cb-thiaminase I mutants and structural data.

D302N mutant is reduced 28-fold. In the next step, the thiazole departs in a reaction assisted by deprotonation of the C4 amino group by Asp94. In support of this, the D94N mutant shows a 1000-fold reduction in k_{cat}. The thiazole departure is also facilitated by Tyr80, and the Y80F mutant shows a 215-fold reduction in k_{cat}. A possible explanation for this effect is that the tyrosine phenol, anchored by hydrogen bonding to Asp94 and Tyr46, holds the thiamin thiazolium in a conformation that maximizes its reactivity as a leaving group with the breaking C−N bond perpendicular to the plane of the pyrimidine. The Tyr80-Tyr46 hydrogen bond is not critical because the Y46F mutant shows only a 4-fold reduction in k_{cat}. Replacement of the thiazolium by a variety of nucleophiles involves the microscopic reverse of these steps. Alignment of thiaminase I sequences from several organisms shows conservation of Cb-thiaminase I residues Tyr80, Asp94, Cys143, Glu271, and Asp302 (Figure S2, Supporting Information). Tyr46 is only partially conserved, consistent with the high mutant activity.

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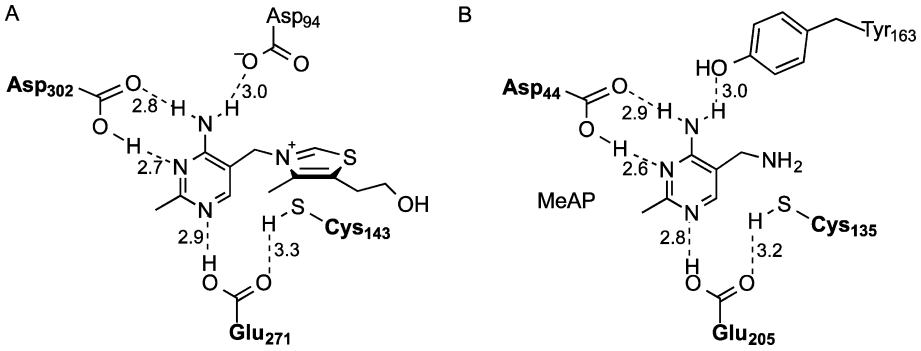

Figure 7. Comparison of active sites for thiaminase I and II. (A) Thiamin binding interactions in Cb-thiaminase I modeled from the C143S/thiamin structure. Ser143 was replaced by cysteine while the χ_1 was kept constant. (B) Model for MeAP binding in thiaminase II (PDB ID: 1YAK). The substrate was generated by replacing the HMP hydroxyl group with an amino group. Functionally conserved residues are labeled in bold font.

Relationship to Thiaminase II. Thiaminase II from *Bacillus subtilis* (also known as TenA) cleaves thiamin into its pyrimidine and thiazole moieties; however, its main physiological function is the conversion of 2-methyl-4-amino-5-aminomethylpyrimidine (MeAP) to 2-methyl-4-amino-5-hydroxymethylpyrimidine (HMP). In contrast to thiaminase I, thiaminase II utilizes only water as the nucleophile.[17] In addition, thiaminase II will not cleave ThMP and ThDP, whereas thiaminase I tolerates modification of the thiazolium moiety and degrades many thiamin analogs.[17,37] Interestingly, like thiaminase I, thiaminase II is often clustered with other enzymes in the thiamin biosynthetic pathway. This grouping of the biosynthetic and degradation enzymes was initially thought to be counterproductive. It was later discovered that thiamin does not appear to be the preferred physiological substrate for *B. subtilis* thiaminase II. Instead, thiaminase II salvages base-degraded pyrimidine from the environment for incorporation into thiamin.[23,44,45]

Thiaminase II adopts an all α-helical fold that shows no sequence or structural homology to the Cb-thiaminase I structure. The active site of thiaminase II is buried, leading to exclusive use of water as the nucleophile. In contrast, the Cb-thiaminase I has a two-domain PBP fold, and an accessible active site location near the surface of the enzyme, thus allowing a variety of nucleophiles to participate in catalysis. No thiaminase II structure has been reported with bound thiamin; however, the structure of *B. subtilis* thiaminase II (PDB ID: 1YAK) with the reaction product HMP reveals the key active site residues.[17]

Despite their major structural differences, thiaminase II and Cb-thiaminase I share some active site features (Figure 7). Both feature a catalytic cysteine that is activated by glutamate, and both use an aspartic acid residue to anchor the pyrimidine through hydrogen bonds to N3′ and the 4′-amino group.[45] In both thiaminase I and thiaminase II, the cysteine side can exist in two conformations.[17,18] In one conformation, the cysteine residue is positioned to be deprotonated by a glutamate side chain. In the other conformation, the cysteine sulfur atom is about 3 Å from the pyrimidine C6 atom. In thiaminase II, the cysteine residue may also require interactions with two intermediate tyrosine residues.[45] Although both thiaminase I and II appear to share a similar catalytic mechanism for thiamin degradation, their major structural differences and substrate preferences demonstrate that they evolved independently and, therefore, may serve different physiological purposes.

We currently differentiate between thiaminase I and thiaminase II by sequence analysis of the entire thiaminase gene. Thiaminase I is rarely found, whereas thiaminase II is abundant. As the active sites of the two enzyme families could, in principle, catalyze either substitution reaction, the possibility remains that some genes annotated as thiaminase II may have thiaminase I activity. However, because thiaminase II appears to accept a variety of modifications to the thiamin pyrimidine moiety, we are not yet able to address this interesting question by analyzing the binding sites of the leaving group.

■ ASSOCIATED CONTENT

Supporting Information

The experimental method for determining the pH rate profile of native Cb-thiaminase I. Supplementary Figure 1 is the pH rate profile for the thiaminase I reaction. Supplementary Figure 2 is a multiple sequence alignment that shows the conserved residues in thiaminase I. This material is available free of charge via the Internet at http://pubs.acs.org.

Accession Codes

‡The coordinates of the C143S/thiamin complex have been deposited in the Protein Data Bank under accession code 4KYS.

■ AUTHOR INFORMATION

Corresponding Author

*T. P. Begley and S. E. Ealick. Address: Department of Chemistry and Chemical Biology, Cornell University, Ithaca, NY 14853. Tel: (607) 255-7961. Fax: (607) 255-1227. E-mail: T. P. Begley, begley@mail.chem.tamu.edu; S. E. Ealick, see3@cornell.edu.

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Notes

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■ ABBREVIATIONS

ACDP, 4-amino-6-chloro-2,5-dimethylpyridimine; PBP, periplasmic binding protein; LB, Luria-Bertani; TEV, tobacco etch virus; NTA, nitriloacetic acid; NE-CAT, Northeastern Collaborative Access Team; RMSD, root mean squared deviation; ThMP, thiamin monophosphate; ThDP, thiamin diphosphate; TPK, thiamin pyrophosphokinase; HMP, 2-methyl-4-amino-5-hydroxymethylpyrimidine; MeAP, 2-methyl-4-amino-5-aminomethylpyrimidine

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