Structure of a eukaryotic thiaminase I

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Thiaminases, enzymes that cleave vitamin B1, are sporadically distributed among prokaryotes and eukaryotes. Thiaminase I enzymes catalyze the elimination of the thiazole ring moiety from thiamin through substitution of the methylene group with a nitrogenous base or sulfhydryl compound. In eukaryotic organisms, these enzymes are reported to have much higher molecular weights than their bacterial counterparts. A thiaminase I of the single-celled amoeboid flagellate *Naegleria gruberi* is the only eukaryotic thiaminase I to have been cloned, sequenced, and expressed. Here, we present the crystal structure of *N. gruberi* thiaminase I to a resolution of 2.8 Å, solved by isomorphous replacement and pseudo-two-wavelength multiwavelength anomalous diffraction and refined to an *R* factor of 0.231 (*R*_{free}, 0.265). This structure was used to solve the structure of the enzyme in complex with 3-deazathiamin, a noncleavable thiamin analog and enzyme inhibitor (2.7 Å; *R*, 0.233; *R*_{free}, 0.267). These structures define the mode of thiamin binding to this class of thiaminases and indicate the involvement of Asp272 as the catalytic base. This enzyme is able to use thiamin as a substrate and is active with amines such as aniline and veratrylamine as well as sulfhydryl compounds such as L-cysteine and β-mercaptoethanol as cosubstrates. Despite significant differences in polypeptide sequence and length, we have shown that the *N. gruberi* thiaminase I is homologous in structure and activity to a previously characterized bacterial thiaminase I.

X-ray crystallography | thiamin degradation

The nonpathogenic unicellular protozoan *Naegleria gruberi* is a ubiquitous eukaryote, found in aerobic and microaerobic environments including freshwater, freshwater silt, and moist soils worldwide (1–4). It exists predominantly as an amoeba but is able to undergo a rapid phenotypic change into a streamlined swimming flagellate when subjected to a nutrient-poor environment (3). *Naegleria* is also able to form resting cysts, which are then capable of excysting back to amoebae (2).

Extracts of *N. gruberi* have been shown to possess a proteinaceous agent that is “cytopathogenic” to vertebrate cells (5). Fulton and Lai show that these extracts of *N. gruberi* amoebae cause generations-delayed apoptotic death of both proliferating and quiescent vertebrate cells in culture (6). The apoptosis-inducing agent was isolated based on activity, and characterized and identified as a thiaminase I, an enzyme that degrades thiamin (vitamin B1) and thiamin diphosphate (TPP), the biologically active form of thiamin (7). They showed that *N. gruberi* thiaminase I induces apoptosis via its enzymatic activity in that an enzymatically inactive mutant lost its ability to cause cell death (7). Active thiaminase I depletes extracellular thiamin, which leads to subsequent depletion of intracellular TPP, an essential coenzyme for many enzymes involved in carbohydrate and energy metabolism, and in turn triggers apoptosis by an as-yet-to-be-characterized mechanism. It has long been known that thiamin deficiency in animals can cause neurological and cardiac symptoms that ultimately lead to death of the animal. In humans, beriberi and Wernicke–Korsakoff syndrome are associated with chronic thiamin deficiency (8). Fulton and Lai have shown that the *N. gruberi* thiaminase I is also capable of killing cells resistant to commonly used chemotherapeutic agents and suggest that thiamin depletion-induced cell death may be a suitable candidate for use in tissue-targeted cancer therapies (6).

Thiaminases catalyze the cleavage of biologically active thiamin into its pyrimidine and thiazole ring moieties (9, 10). These enzymes can be grouped into two classes defined by the nucleophile used in the mechanism by which the cleavage is accomplished. The thiaminase II (EC 3.5.99.2) class of enzymes exclusively uses water to accelerate the hydrolysis of thiamin into 2-methyl-4-amino-5-hydroxymethylpyrimidine (HMP) and 4-methyl-5-(2-hydroxyethyl)thiazole and is found only in bacteria, fungi, and yeast (11). The thiaminase I (EC 2.5.1.2) class of enzymes uses a variety of aromatic and heterocyclic amines and sulfhydryl compounds as substituting bases in a nucleophilic displacement reaction on the methylene group of the pyrimidine moiety (10, 12–15). Thiaminase I is found in specific species of microorganisms such as *Bacillus* and *Clostridium* as well as multicellular organisms including certain ferns, insects, shellfish, and freshwater and ocean fish (10, 14, 16–26). Although distributed throughout the kingdoms, the phylogenetic distribution of thiaminase I, unlike most other enzymes, appears to follow no easily discernable evolutionary pattern. A physiological role has yet to be assigned for thiaminase I (27), and several studies report that thiaminase I enzymes from eukaryotic organisms are produced as larger holoenzymes, 55–200 kDa, some of which are active as smaller fragments (17, 21–23, 25, 26). The presence of this enzyme in the diet of animals can have several deleterious effects and can even be fatal. It is responsible for thiamin deficiency and early mortality syndrome in Great Lakes salmonines, polioencephalomalacia in sheep, and cerebrocortical

Significance

Thiaminases, enzymes that cleave vitamin B1 into its pyrimidine and thiazole ring moieties, are sporadically distributed among prokaryotes and eukaryotes. Thiaminase I enzymes accomplish this reaction through substitution of the thiazole ring with a nitrogenous base or sulfhydryl compound. A thiaminase I of the single-celled amoeboid flagellate *Naegleria gruberi* is the first eukaryotic thiaminase I to have been examined structurally. The crystal structures in both *apo* form and bound to 3-deazathiamin, a noncleavable thiamin analog and inhibitor of the enzyme, define the mode of thiamin binding to this class of thiaminases and indicate the residues important for catalysis. Comparison with thiaminase II argues for convergent evolution between these two enzymes.

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Data deposition: The atomic coordinates and structure factors have been deposited in the Research Collaboratory for Structural Bioinformatics Protein Data Bank, www.rcsb.org [ID codes 4HCW (*apo* Ng-thiaminase) and 4HCY (Ng-thiaminase bound to 3-deazathiamin)].

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Fig. 1. The gene that encodes the thiaminase I examined in this study yields a protein with homology to both thiaminase I and transketolase enzymes. Key active-site residues are indicated.

necrosis in cattle (9, 28, 29). Consumption of silk worm larvae as a source of protein in Nigeria causes acute seasonal cerebellar ataxia (22). Finally, the 1860–1861 expedition of Burke and Wills across Australia turned fatal when the men, whose diet consisted primarily of raw nardoo fern, suffered from thiaminase poisoning, developed beriberi, and died (30). It is tempting to speculate that these organisms have acquired thiaminase I through horizontal gene transfer and maintained it in their genomes as a mechanism of defense.

The *N. gruberi* thiaminase I is part of a large polypeptide sequence of ~1,025 aa residues (D2V4Z5; Fig. 1). The C-terminal portion of this sequence (669 aa) is homologous to transketolases. It is 49% identical in sequence to the *Saccharomyces cerevisiae* transketolase (P23254) and includes conservation of key residues involved in dimer stabilization, cofactor binding, and catalysis in the yeast enzyme. Attempts to isolate the full-length protein from extracts of *N. gruberi* amoebae have been unsuccessful; however, the thiaminase I activity survives, indicating that the thiaminase I domain of the protein remains intact. In addition, the N-terminal domain expressed by itself (356 aa) has thiaminase I activity in vitro that is comparable to the activity of the thiaminase I domain isolated from cellular extracts (6).

Given the low sequence identity (~25%) of the thiaminase I isolated from *N. gruberi* (Ng-thiaminase) to the previously characterized *Bacillus thiaminolyticus* (*Paenibacillus thiaminolyticus*) thiaminase I (P45741) (Bt-thiaminase) and the fact that the *N. gruberi* enzyme is part of a larger protein, we endeavored to determine its structure. Additionally, the sequence of the Bt-thiaminase enzyme contains only one cysteine, which is located in the active site, whereas the sequence of the Ng-thiaminase enzyme possesses six cysteines. The structure of the Bt-thiaminase has been solved bound to a mechanism-based inhibitor (31), 4-amino-6-chloro-2,5-dimethylpyrimidine (Pvd) (Fig. S1); however, this molecule represents only the pyrimidine portion of thiamin and may not be representative of the mode of substrate binding to this class of thiaminases. Here, we describe the X-ray crystal structures of a thiaminase I from *N. gruberi* in its unliganded state and bound to a substrate analog, 3-deazathiamin (3-dzThi) (Fig. S1), which is isoelectronic with and essentially identical in shape and size to thiamin (32, 33). The *N. gruberi* thiaminase I has been shown to use thiamin, but not thiamin diphosphate, as a substrate and is active with both amines and sulfhydryl compounds as cosubstrates. Despite low sequence identity and differences in native polypeptide length, the tertiary structure of the thiaminase I from *Naegleria gruberi* closely resembles that of thiaminase I from *Bacillus thiaminolyticus*.

Results

Overall Structure. The structure of Ng-thiaminase was solved using a combination of isomorphous replacement, pseudo-

multiwavelength anomalous diffraction phasing, and molecular replacement (Table S1). Both the *apo* and *holo* structures of Ng-thiaminase crystallize with six molecules in the asymmetric unit. The Ng-thiaminase has two globular domains of similar size and α/β topology, each consisting of a mixed β -sheet sandwiched by α -helices, separated by a deep cleft at the domain interface (Fig. 2). The N-terminal domain consists of a seven-stranded β -sheet surrounded by eight α -helices and the C-terminal domain contains a four-stranded β -sheet flanked by eight α -helices. Numbering of the protein begins with the N-terminal methionine of the actual protein sequence. The first four residues at the N terminus were not observed in the crystal structure. Amino acid residues 5–118 and 260–315 lie within the N-terminal domain, and the C-terminal domain consists of residues 119–259 and 316–356. Three peptide segments consisting of residues 118–120, 258–260, and 328–333 connect the domains. The first two segments comprise a two-stranded crossover region that forms the back of the cleft between the domains. Comparison of α -carbon atoms using LSQMAN (34) indicates that all copies of the molecule in the asymmetric unit are essentially identical for both the *apo* (average rmsd, 0.13 Å) and *holo* (average rmsd, 0.05 Å) crystal structures.

For both the *apo* and *holo* structures of Ng-thiaminase, the contents of the asymmetric unit pack in such a way that three dimer pairs participate in a threefold screw axis where one monomer in each layer is also related by twofold symmetry to one of the monomers in the neighboring layer. This crystal packing suggests the enzyme could be a monomer or a dimer, but gel filtration experiments show that in solution Ng-thiaminase is a monomer. The Bt-thiaminase is also a monomer.

Active Site. The assignment of the active site to a deep cleft between the N- and C-terminal domains is confirmed by the location of Cys118, predicted by sequence alignment with Cys113 of Bt-thiaminase to be the active site cysteine (Fig. 2 and Fig. S2). More importantly, clear electron density for one 3-dzThi molecule within this binding site for all monomers in the asymmetric unit is observed (Fig. S3). The overall character of the active site is conserved relative to the Bt-thiaminase structure (Fig. 3B). Of the 11 residues that make up the bulk of the Ng-thiaminase active site, 8 are identical in both thiaminases (Tyr15, Tyr53, Asp68, Cys118, Tyr230, Glu232, Tyr260, Asp262) and 1 is a conservative substitution of a Phe (Phe13) for a Tyr residue. The active site of Ng-thiaminase contains two aromatic active-site residues, Tyr116 and Phe158, with smaller side-chain equivalents (Ile111 and Thr163) in Bt-thiaminase. There is no structural equivalent in Ng-thiaminase for Tyr222 in Bt-thiaminase; it resides on the loop between α 10 and α 11, which is absent in Ng-thiaminase (Fig. S2). The predominance of aromatic residues

Fig. 2. Stereoview ribbon diagram of the *N. gruberi* thiaminase I bound to 3-dzThi, a substrate analog (PDB ID code 4HCY). α -Helices are red and β -strands are blue. 3-dzThi, Glu241, Cys118, and Asp272 are green.

Kinetics. The Ng-thiaminase has been purified and characterized in vitro and possesses unusual stability for an enzyme. It has a half-life of 8–10 mo when stored at 0–4 °C and of 1 wk when incubated at 37 °C (6). It is also remarkably resistant to proteolysis as the thiaminase I portion of the larger polypeptide (Fig. 1) can be isolated in its active form from whole-cell *N. gruberi* lysates (6). The Ng-thiaminase uses thiamin and accepts a number of heterocyclic amines and sulfhydryl compounds as the nucleophile in the cleavage reaction, including aniline, veratrylamine, β -mercaptoethanol (β -ME), and L-cysteine (Fig. S1). By definition, the enzyme is therefore considered a thiaminase I. The kinetic

In an effort to find compounds that would show how the substrate binds, analogs of thiamin were tested for activity. Oxythiamin (Fig. S1), with which the enzyme reacts weakly in the

presence of 1 mM veratrylamine, has a millimolar inhibition constant. However, 3-dzThi does not react at all, was shown to act as a competitive inhibitor with a micromolar inhibition constant, and could be used as an isosteric inhibitor to indicate the mode of binding of the substrate. Consequently, 3-dzThi was used to obtain cocrystals for structural analysis.

Discussion

Overall Structure. A search for structurally similar proteins using the DaliLite server (39) revealed, as expected, that the overall fold of Ng-thiaminase is very similar to that of the Bt-thiaminase [Protein Data Bank (PDB) ID code 2THI] (31) and to members of the group II periplasmic-binding protein family (40, 41), such as the trehalose/maltose-binding protein (PDB ID code 1EU8) (42). One striking difference in the structures of these two enzymes is the insertion of an antiparallel, two-stranded “ β -finger” between $\beta 1$ and $\alpha 1$ in the N-terminal domain of the Ng-thiaminase structure that corresponds to an insertion in the N-terminal half of the Ng-thiaminase polypeptide (Fig. S2). A sequence insertion in the C-terminal half of the Bt-thiaminase sequence yields a loop of similar size, but lacking secondary structure, also in the N-terminal domain but between $\alpha 14$ and $\alpha 15$. The location of the insertion in the Ng-thiaminase sequence moves the tip of the finger closer to the opening of the active site cleft by ~ 19 Å relative to the unstructured loop observed in the Bt-thiaminase structure. The active site in the Bt-thiaminase structure is partially shielded by a loop located between $\alpha 10$ and $\alpha 11$, which is lacking both in the sequence and structure of the Ng-thiaminase protein.

It is tempting to assign a role to the Ng-thiaminase β -finger, and the corresponding unstructured loop in the Bt-thiaminase structure, in regulating access to the active site. However, there is no indication of a major conformational change in Ng-thiaminase upon binding of the substrate analog 3-dzThi, implying a lock-and-key mechanism over an induced-fit mechanism. The α -carbon atoms in the *apo* and *holo* structures can be superimposed to give an rmsd of 0.23 Å. A similar observation can be made when comparing the *apo* and Pvd inhibitor-bound structures of Bt-thiaminase (rmsd, 0.51 Å). For comparison, it has been demonstrated that many members of the periplasmic binding protein superfamily (40) undergo a significant hinge motion upon ligand binding. For example, the maltose binding protein undergoes a hinge motion of 35° (43). Excluding the constraints of crystal packing, one possible explanation for this difference lies in the different function of the periplasmic binding proteins compared with the thiaminase I enzymes. A large hinge motion may be more advantageous to a protein that binds to, transports, and releases a ligand, than to a protein that binds a ligand and then performs catalysis.

Although gel filtration experiments indicate that the Ng-thiaminase is a monomer in solution, the crystallized protein can be interpreted in terms of potential oligomeric structures (Fig. S4). The portion of the polypeptide sequence not included in the Ng-thiaminase clone is homologous to transketolases, which are biologically active dimers with N and C termini arranged so that they are located on the same surface of the protein. Both of the potential Ng-thiaminase dimers observed in the asymmetric unit are arranged so that the N and C termini of each monomer would be on the same surface of the dimer. Either potentially biologically relevant dimer could allow for the full-length protein to interact in such a way so that the transketolase domain could adopt its expected quaternary structure. There are no residues from either active site that interact with the alternate subunit in the dimer. Nevertheless, the extent of buried interface for these potential dimers is significant, and could represent dimeric interactions in the full-length protein.

Substrate Binding. The structure of Ng-thiaminase bound to the substrate analog 3-dzThi confirms the location of the active site within a cleft between the N- and C-terminal domains (Fig. 2) and identifies the residues involved with substrate binding (Fig.

3). The predicted active-site residue, Cys118, is located deep within this cleft and, when mutated to a serine, yields a protein that tests negative for enzymatic activity (7). Glu232 is also located in the active site and is hydrogen bonded to the substrate analog. Although one of the Glu232 side-chain carboxyl oxygen atoms interacts with N1' on 3-dzThi, the other is positioned ~ 3.3 Å from the sulfhydryl group of Cys118. This glutamate is equivalent to Glu241 in the Bt-thiaminase, which, when mutated to glutamine, has been shown to be inactive (31).

In the Ng-thiaminase/3-dzThi complex and the Bt-thiaminase/Pvd complex, the two pyrimidine rings take on very different configurations, presumably dictated by the potential interactions open to the respective compounds (Fig. 3B). For instance, a greater number of hydrogen-bonded contacts are made between the pyrimidine ring of 3-dzThi and active-site residues than are observed in the Bt-thiaminase/Pvd complex (31). The orientation of the pyrimidine moiety of 3-dzThi bound to Ng-thiaminase is approximately perpendicular to the orientation of Pvd in complex with the Bt-thiaminase complex, with the exocyclic N4' nitrogens pointing in opposite directions. In the Ng-thiaminase/3-dzThi structure, this orientation brings N4', N3', and N1' into position to form hydrogen bonds with Asp262 and Glu232. None of the analogous interactions are observed in the Bt-thiaminase/Pvd complex, but residues structurally equivalent to Tyr53, Asp68, Cys118, Tyr230, Glu232, Tyr260, and Asp262 in Ng-thiaminase are present and would be available to interact with thiamin in a similar manner. The orientation of the Pvd moiety in the Bt-thiaminase complex is presumably dictated by the covalent bond between Cys113 and C6' of the inhibitor, whereas the orientation of the pyrimidine ring in the Ng-thiaminase/3-dzThi complex is dictated by the presence of the thiazole ring, which, together with the hydroxyl group, lies closer to the lip of the active site. The thiazole ring of 3-dzThi is sandwiched by a hydrophobic channel made up of Tyr15, Tyr53, Phe158, and Tyr230, and is effectively held in place. Together, these interactions orient the pyrimidine ring precisely in the active site so that Glu232 is in position to accept a proton from Cys118 when or if the sulfur makes a covalent bond to the C6' of the pyrimidine moiety as part of the reaction mechanism. The relevance of this orientation is supported by the previously proposed mechanism for Bt-thiaminase in which the glutamate serves as a base for activation of the active-site cysteine (31).

Unlike many enzymes that respond structurally to the presence of an inhibitor, especially one as isosteric to the substrate as this one, the active site of Ng-thiaminase in the presence of 3-dzThi is remarkably similar to the *apo* structure (Fig. 3B). Although some shifts in side-chain positions are observed for Cys118 and Glu232, the largest shifts in side-chain positions are observed for Phe158, Tyr230, and Tyr260. The side chains of these aromatic residues move closer to the aromatic parts of the inhibitor, forming tighter interactions. This is in contrast to the active site of Bt-thiaminase (31), where there is a larger shift in active-site residues between the *apo* and inhibitor complex structures. However, it is not clear whether these differences are relevant because the Pvd inhibitor does not seem to mimic the structure of the substrate very well.

The conformation of thiamin bound to the active site of an enzyme determines whether it is a substrate or a cofactor. The conformation of thiamin is defined by the dihedral angles ϕ_T (C5'-C3,5'-N3-C2) and ϕ_P (N3-C3,5'-C5'-C4') (44). In enzymes that use TPP as a cofactor, the higher energy “V” conformation (approximately $\phi_T = 104^\circ$, $\phi_P = 64^\circ$) is observed, whereas, in the periplasmic thiamin-binding protein, thiamin-monophosphate is bound in the low-energy “F” conformation (PDB ID code 2QRY; $\phi_T = 1^\circ$, $\phi_P = -82^\circ$) (45). The conformation of 3-dzThi is more similar to the “F” conformation (monomer A, $\phi_T = -24.72^\circ$, $\phi_P = -103.26^\circ$), which places the N4' amino group of the pyrimidine ring out of proximity to C2 on the thiazole ring. In this conformation, the 4'-amino group on the pyrimidine ring cannot act as a base. Additionally, the Ng-thiaminase does not

Materials and Methods

Detailed methods are given in *SI Materials and Methods*. Recombinant Ng-thiaminase was expressed in and purified from an *Escherichia coli* expression system using an N-terminal enterokinase cleavable His-tag. Crystallization, X-ray data collection, and subsequent structure determination were carried out on Ng-thiaminase from which the affinity tag was removed. Thiaminase activity assays were performed using tagged protein.

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