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Mutagenesis studies on TenA: A thiamin salvage enzyme from *Bacillus subtilis*

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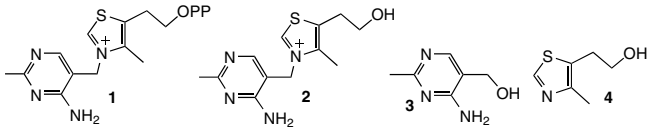
Abstract

TenA catalyzes the hydrolysis of 4-amino-5-aminomethyl-2-methylpyrimidine and participates in the salvage of base-degraded thiamin. Here, we describe mutagenesis of the active site of TenA guided by structures of the enzyme complexed to a substrate analog and to the product. Catalytic roles for each of the active site residues are identified and a mechanism for the reaction is described.

Keywords: TenA; Thiaminase II; Mechanism; Structure; Mutagenesis; Thiamin degradation

1. Introduction

Thiamin pyrophosphate **1** is an essential cofactor in all living systems and is biosynthesized by a complex pathway [1,2]. Consequentially, many microorganisms also contain salvage pathways for thiamin fragments present in the environment and salvage pathways for thiamin alcohol **2** [3], hydroxypyrimidine **3** [4], thiazole **4** [5] and base-degraded thiamin **5** [6] have been identified.



The salvage of base-degraded thiamin is described in Fig. 1. In this pathway, formyl aminopyrimidine **5**, formed by base-mediated degradation of the thiazolium moiety of thiamin, is transported into the cell, deforymylated and the resulting aminopyrimidine **6** is converted to hydroxypyrimidine **3** by 4-amino-5-aminomethyl-2-methylpyrimidine hydrolase (TenA, thiaminase II) [6]. The salvaged hydroxy-pyrimidine (3) is then phosphorylated and incorporated into the *de novo* thiamin biosynthetic pathway.

The crystal structure of the aminopyrimidine hydrolase (TenA), with hydroxy-pyrimidine **3** bound at the active site, has recently been reported [7]. Here, we describe a systematic mutagenesis study of the key active site residues identified from this structure. This information is used to propose a detailed mechanism for the TenA-catalyzed aminopyrimidine hydrolysis.

2. Methods and materials

2.1. Purification of TenA

TenA was cloned, overexpressed and purified as described previously [7].

2.2. Preparation of TenA mutants

Standard methods were used for DNA manipulations. Plasmid DNA was purified with the Qiagen Miniprep kit. *Escherichia coli* strain MachI (Invitrogen) was used as a recipient for transformations during plasmid construction

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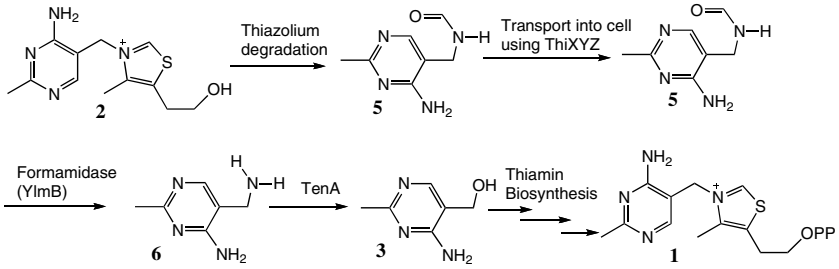

Fig. 1. The salvage pathway for base-degraded thiamin.

Table 1
Mutagenesis primers

Mutant	Primer sequence, top strand	Restriction site
D44A	CCGTTTTAAATACTACGTACTTCAAGCTTCCTATTATTTAACGC	HindIII
C135A	GCGGCCCTGCTGCCCGCGTATTGGCTCTATTACGAGG	BstXI
E205A	GTCATCTCCAGCTACTATGCATATCAATTTTGGGGAATGG	NsiI
Y47F	CTTCAGGATTCCTATTTTCTGACGCATTTTGCAAAGG	HgaI
Y112F	CCTACGGCGTACTCTTTTACGTCCCATATGTACC	BsmFI

and for plasmid propagation and storage. Site-directed mutagenesis was performed on the TenA overexpression plasmid (pBsTenA.XF1) by a standard PCR protocol using *Pfu*Turbo DNA polymerase per the manufacturer's instructions (Invitrogen) and *Dpn*I (New England Biolabs) was used to digest the methylated parental DNA prior to transformation. Primers were designed to introduce or remove a diagnostic restriction enzyme site to facilitate screening for a mutated clone. Only clones producing the anticipated restriction pattern were sequenced. The mutagenesis primer pair consisted of the primer whose sequence is in Table 1 and its reverse complement.

2.3. Enzymatic assay

The enzymatic hydrolysis of aminopyrimidine **6** (gift from Roche) was monitored using the glutamate dehydrogenase assay for ammonia [8]. Each reaction mixture contained five units of glutamate dehydrogenase (unit is defined as the amount of glutamate dehydrogenase that will reduce 1 μmol of α-ketoglutarate to glutamate per min at pH 8.3 at 30 °C), 5 mM α-ketoglutarate, 0.1 mM EDTA, 250 μM NADPH, varing concentrations of aminopyrimidine **6** and TenA, which was added last to initiate the reaction. The TenA concentration was determined from its calculated extinction coefficient at 280 nm of 74,280 $M^{-1}\,cm^{-1}$ (determined by the Edel-Hoch Method [9]). NADPH consumption was monitored by measuring the decrease in absorbance at 340 nm. K_m and k_{cat} were obtained by fitting the initial rates, at varying substrate concentrations (determined at less than 10% conversion), to the Michaelis–Menten equation using the program GraFit. These parameters for the native and mutated enzyme are shown in Table 2. The K_m for Y112F and Y47F could not be directly measured because of the low activity of these mutants.

Table 2
Kinetic parameters for TenA and its active site mutants

Protein	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($min^{-1}\,\mu M^{-1}$)	Relative k_{cat}/K_m
Native	11.8 ± 1.6	22 ± 0.5	1.9 ± 0.3	1
E205A	251 ± 31.5	0.27 ± 0.006	0.001 ± 0.0001	0.0005
D44A	337 ± 94	0.09 ± 0.004	0.0003 ± 0.00008	0.0003
Y112F	<25	0.31 ± 0.02	>0.012 ± 0.0007	>0.01
Y47F	<25	0.75 ± 0.03	>0.029 ± 0.0012	>0.03
C135A	Inactive	Inactive	Inactive	Inactive

3. Results and discussion

TenA catalyzes the substitution of the amino group of amino-pyrimidine **6** by water (Fig. 1). This reaction is analogous to the well-studied thiaminase I-catalyzed substitution of the thiamin thiazole by a variety of nucleophiles which occurs by an addition elimination mechanism (Fig. 2). In this mechanism, cysteine adds to C6 of the pyrimidine generating anion **7**. Expulsion of the leaving group gives **8**. Addition of the nucleophile (X–H), followed by expulsion of the active site cysteine completes the reaction. This mechanistic proposal is supported by the identification of the active site nucleophile as Cys113 [10], the observation of ping-pong kinetics [11], retention of stereochemistry [12], and a structure of the enzyme with an analog of **7** bound at the active site [13].

The structure of TenA with bound product is shown in Fig. 3A [7]. The location of Cys135 close to C6 of the pyrimidine (3.2Å) suggested that the mechanism of TenA was likely to be similar to the mechanism of thiaminase I. To test this, key residues surrounding the active site

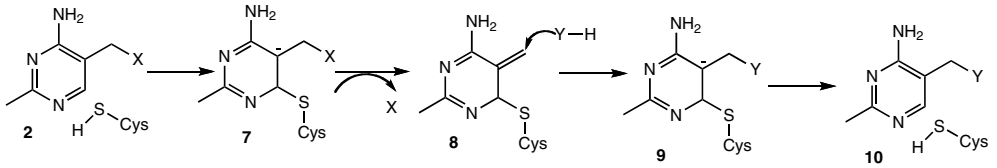

Fig. 2. Mechanistic proposal for the Thiaminase I-catalyzed reaction. X represents the thiamin thiazole and Y represents a variety of nucleophiles.

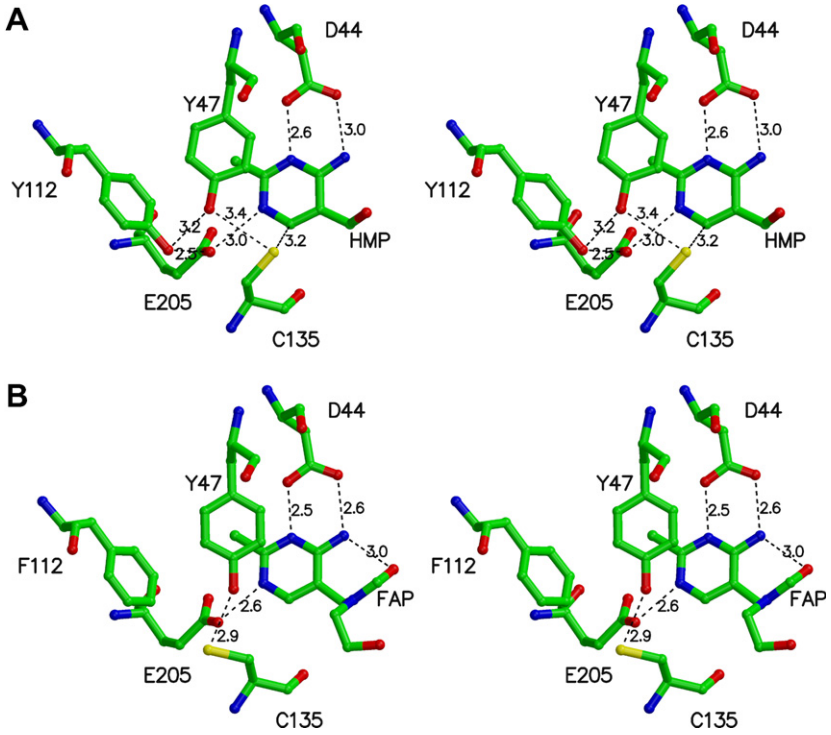

Fig. 3. Active site structure of TenA. (A) Native enzyme complexed with 4-amino-5-hydroxymethyl-2-methylpyrimidine (PDB 1YAK). (B) The Y112F mutant complexed with 4-amino-5-(*N*-formyl-*N*-hydroxyethyl)aminomethyl-2-methypyrimidine (PDB 2QCX).

pyrimidine were altered by mutagenesis to probe their function.

The results of these mutagenesis experiments are shown in Table 2 and are consistent with the mechanistic proposal outlined in Fig. 4. In this mechanism, Cysteine 135 adds to the pyrimidine and the resulting pyrimidine anion is stabilized by protonation at N1 by glutamic acid 205 to give **12**. The observation that the C135A mutant is inactive and that the E205A mutant shows a 2000-fold reduction in catalytic activity is consistent with this. Cysteine 135 is activated by deprotonation using a catalytic triad consisting of Tyrosine 47, Tyrosine 112 and glutamic acid 205. Consistent with this, the Y112F mutant showed a 71-fold reduction in k_{cat}, and the Y47F mutant showed a 29-fold reduction in k_{cat}. Both mutants showed less than 2-fold increase in K_m.

The structure also suggests that aspartic acid 44 anchors the substrate at the active site and further polarizes the pyrimidine for cysteine addition. The D44A mutant shows a 3300-fold reduction in catalytic activity. Loss of ammonia from **12** gives **13**. Surprisingly, the amine leaving group does not interact with any of the active site amino acid side chains. Reversal of these steps, using water as the nucleophile, completes the reaction (**14**–**16**).

TenA is involved in the salvage of the thiamin pyrimidine from base-degraded thiamin (Fig. 1). Formyl aminopyrimidine **5** is not a substrate for TenA. To better understand the substrate selectivity of TenA, the structure of the Y112F mutant, complexed to 4-amino-5-(*N*-formyl-*N*-hydroxyethyl)aminomethyl-2-methylpyrimidine was determined (Fig. 3B). This structure shows that this analog binds in a catalytically competent manner and that the enzyme also does not provide any acidic residues to stabilize an amide leaving group. Under these conditions, the substrate selectivity is most likely due to ammonia being a better leaving group than an amide anion (relative pK_a values of 9.2 and 17, respectively).

Thiaminase I and TenA both catalyze pyrimidine substitution reactions at the C5′ of the thiamin pyrimidine. These enzymes share no sequence or structural similarity [7], yet both enzymes utilize a similar addition–elimination mechanism. In contrast, thiamin phosphate synthase catalyzes the substitution of the pyrophosphate of **17** by thiazole phosphate using a dissociative mechanism involving a pyrimi-

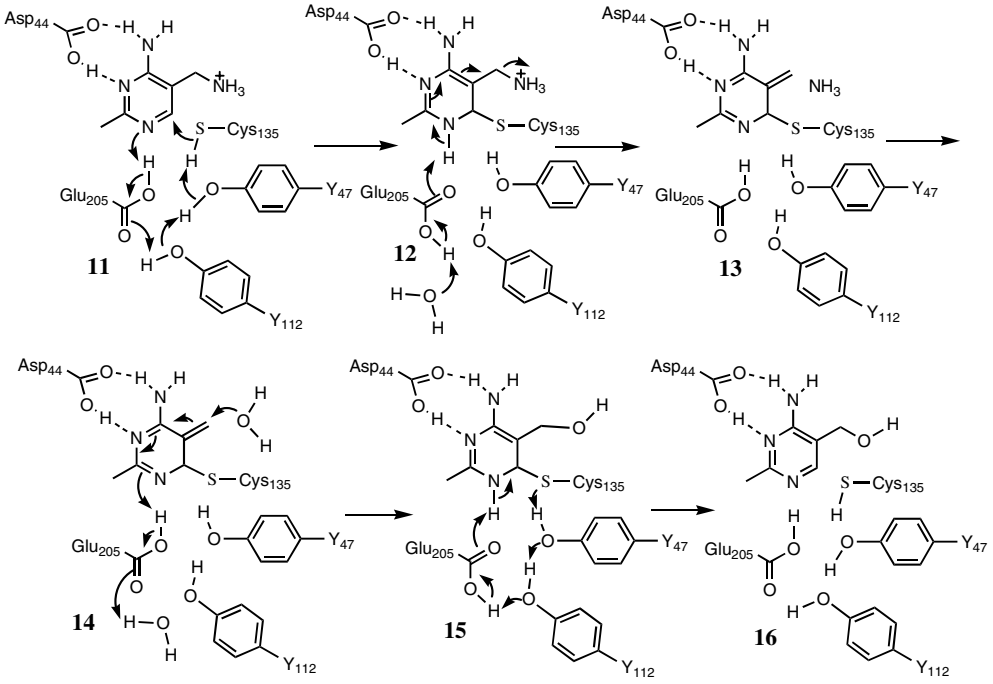

Fig. 4. Mechanistic proposal for the TenA-catalyzed cleavage of aminopyrimidine **6**.

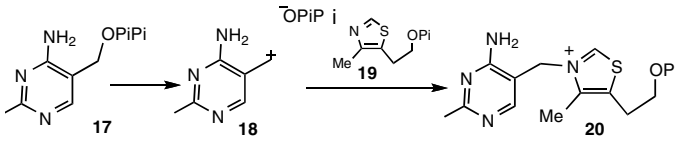

Fig. 5. The thiamin phosphate synthase catalyzed reaction proceeds via a dissociative mechanism.

dine carbocation intermediate (Fig. 5) [14]. A comparison of the mechanisms of these three enzymes suggests that substitution at C5′ of the thiamin pyrimidine occurs by a dissociative mechanism when the leaving group is good (e.g. pyrophosphate) and by an addition–elimination mechanism when the leaving group is poor (e.g. thiazole or ammonia). In contrast, benzylic substitution reactions proceed by associative (SN2) and dissociative (SN1) mechanisms depending on reaction conditions and on the nucleophile and leaving group. The addition–elimination mechanism is not observed for benzylic substitution because of the high stability of the 6π electron system in benzene which is disrupted by nucleophilic addition. The benzene ring also lacks the basic nitrogen atoms required to polarize the pyrimidine for nucleophilic addition.

Acknowledgments

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