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Crystal Structure of a Flavin-dependent Thymidylate Synthase from *Helicobacter pylori* strain 26695

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Abstract: ThyX, a flavin-dependent thymidylate synthase that is involved in the synthesis of dTMP from dUMP, is a promising target for the development of novel antibacterial drugs that aimed at blocking the biosynthesis of dTMP, one of the building blocks of DNA. This enzyme has been recently identified in some dsDNA viruses and pathogenic bacteria, including the gastric pathogen *Helicobacter pylori*. It shares neither sequence nor structural homology with the classical ThyA in humans and other organisms. Further more, ThyX and ThyA are the only source of dTMP in these organisms and other pathways cannot substitute for their function. Thus, ThyX-specific inhibitors could be effective antibacterial reagents while having no impact on human cells. Here we report the crystal structure of ThyX from *Helicobacter pylori* strain 26695 in complex with co-factor FAD and substrate dUMP at 2.5 Å resolution, which consists of a 1.5 tetramer of ThyX with a total of 1248 residues, six FAD and six dUMP molecules in an asymmetric unit. The structure revealed the key residues that are involved in co-factor FAD and substrate dUMP binding, site-directed mutagenesis were performed to analysis the importance of these residues on ThyX activity by genetic complementation and FAD binding assay.

Keywords: Crystal structure, dUMP, FAD, Flavin-dependent thymidylate synthase, *Helicobacter pylori*.

INTRODUCTION

Helicobacter pylori (*H. pylori*) infects more than 50% of the world population and is a major cause of gastritis and peptic ulcers and is a risk factor for gastric cancer[1]. Eradicating the bacterium has been suggested to be important for curing these associated diseases [2, 3], and several antimicrobial agents are used to eradicate the bacterium [4, 5]. However, because of the emergence of antimicrobial resistant strains, it has become increasingly difficult to eradicate the pathogen, resulted in treatment failure and disease recurrence [6]. Thus, the discovery and selection of new targets for antibiotic development are urgently needed.

Of the four building blocks of DNA, dATP, dCTP, and dGTP can be produced directly by ribonucleotide reductases from the corresponding ribonuclotides, whereas dTTP is synthesized *de novo* via reductive methylation of 2'-deoxyuridine-5'-mono -phosphate (dUMP) to forms 2'-deoxythymidine-5'-monophosphate (dTMP) and which is subsequently phosphorylated to form the final product dTTP[7]. In most eubacteria and eukaryotic cells, the synthesis of dTMP from dUMP is catalyzed by thymidylate synthase (TS, EC 2.1.1.45), which uses N^5,N^{10}-methylene-5,6,7,8- tetrahydropolate (CH_2H_4folate) as a donor of both a methylene group and a hydride [8, 9]. Organisms that are dependent on this pathway invariably contain the genes for both enzymes, *i.e.*, *thyA* (designated *TYMS* in mammals) for TS and *folA* for dihydrofolate reductase.

It had been assumed that the ThyA proteins and the biosynthesis of thymidylate were conserved in all organisms. Therefore, the discovery that both the *thyA* and *folA* genes were missing from the genome sequences of many microorganisms was very puzzling [10]. In 2002, Myllykallio *et al.* demonstrated that an alternative enzyme ThyX from *H. pylori* has *in vivo* and *in vitro* thymidylate synthase activities, and the gene *thyX* functionally replace *thyA* in *Escherichia coli* dTMP synthesis. ThyX is present in several human pathogens, and it shows neither sequence nor structural homology to the classic ThyA proteins [11, 12]. Furthermore, the *H. pylori* ThyX protein utilizes a mechanism that is distinct from that of ThyA [12]. The crystal structure of a homologous ThyX protein from *Thermotoga maritime* corroborates the distinction between ThyX and ThyA proteins [13].

Interestingly, ThyX enzymes are only found in microorganisms, especially some of the most important pathogenic bacteria, such as *H. pylori* and *M. tuberculosis*. The fact that ThyX enzymes share no sequence or structural homology [13, 14]with classical TS and that homologous enzymes are absent in humans makes ThyX a promising target for the development of novel antibacterial drugs [15, 16]. For this purpose, it is essential for us to understand the relationships between the enzymatic mechanisms and crystal structures of these proteins; we therefore have embarked on the structure determination of ThyX from *H. pylori*. The gene encoding *H. pylori* ThyX was cloned; the protein was expressed, purified and crystallized; and a diffraction data set was collected

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at 2.50 Å resolution, as described previously [17]. During the time we are screening for potential inhibitors for *H. pylori* ThyX, Wang *et al* [18] has reported the structure of ThyX form *H. pylori* strain SS1, which was highly similar to our structure. So in this study, we report the structure determination of ThyX from *H. pylori* strain 26695 and compare it with homologous structures.

METHODS

1. Structure Determination and Refinement

Using the homologous structures from *T. maritima* (PDB code 3G4A) [13] and *M. tuberculosis* (PDB code 2AF6) [19] as searching models, a solution was found using the program PHASER [20] in which 1.5 tetramers, or 6 ThyX monomers, were located in an asymmetric unit. However, the phases calculated were of poor quality and did not permit model refinement. The phases were improved by following the protocol described by Keller *et al*. [21]. In brief, we first ran 100 cycles of six-fold non-crystallographic symmetry (NCS) averaging with solvent flattening using DM [22] with diffraction data to 5 Å resolution. The masks and NCS operations were refined periodically during the DM run. Then, 1000 cycles of NCS averaging with phase extension in resolution steps and solvent flattening were performed. The six-fold NCS averaging proved to be very effective in removing the phase bias of the molecular replacement model, and the overall figure-of-merit was refined to 0.834 with all the reflections to 2.5 Å resolution. The electron density calculated with the refined phase was of excellent quality and was used for automatic model building using the program Arp/wArp [23]. The program successfully docked 87% (1126 out of 1296 residues from 6 ThyX molecules) of the residues into the density map. The remaining residues and complexed FAD and dUMP moieties were built into the model manually using the program COOT [24]. The model was then subjected to alternative cycles of refinement using the program CNS [25] and model adjustment using COOT. The final model has R_{work} and R_{free} factors of 19.5% and 24.3%, respectively. Residues 1-23 from all 6 ThyX molecules were missing. All six chains could be traced continuously from residue 24 to 231 in the electron density map. The structure also contains 538 water molecules and 11 sulphate anions. The co-factor FAD and substrate dUMP were well defined in all six subunits of the asymmetric unit. The statistics of data collection and structural refinement are summarized in (Table **1**).

2. Site-directed Mutagenesis

Site-directed mutagenesis was used to introduce mutations into plasmid pET22b-ThyX for several key residues involved in co-factor FAD and substrate dUMP binding. All mutants were generated by DpnI mediated site-directed Mutagenesis [26]. In brief, the mutanted plasmid was amplified by PCR using a pair of primers bearing the mutation at the center. The template is then digested by *DpnI*, a restriction enzyme which cleaves only methylated DNA, while the mutated plasmid is preserved because it was generated *in vitro* and is unmethylated as a result. A total of five mutants, including residues H98K, R109A, S107A, S108A, and R197A, were prepared and assayed, as summarized in (Table **2**). The presence of the mutation was confirmed by DNA sequencing.

Table 1. Refinement Statistics for *H. pylori* ThyX

Refinement Statistics	
Reflections (working/test)	48588/5207
Resolution (Å)	55.63-2.50
R_{work} (%)	19.5
R_{free} (%)	24.3
Protein atoms	10181
Water molecules	538
Sulfate ion	11
FAD	6
dUMP	6
R.m.s.d. bonds (Å)	0.0076
R.m.s.d. angles (°)	1.334
Mean *B*-factor (Å ^{2})	48.425
Ramachandran plot statistics (%)	
Most favored regions	91.1
Additionally allowed regions	8.8
Generally allowed regions	0.1
Disallowed regions	0

Table 2. Genetic Complementation and FAD Binding Activity of Mutations Created in *H. pylori* ThyX

H. pylori ThyX Allele	Complementation Activity	FAD Binding Activity
Wild type	++++	++
H98K	_	_
R109A	_	+
S107A	_	++
S108A	+++	++
R197A	_	+

3. Genetic complementation Assay

An *E. coli* strain lacking endogenous ThyA activity, designated thymidine auxotroph DH5α (ΔthyA::Erm) was used for complementation assay [27]. The DH5α (ΔthyA) strain was transformed with each pET22b-ThyX vector, containing wild-type or mutated ThyX. Transformants were selected on LB agar plates with 100 μg/ml of erythromycin and 100 μg/ml of ampicillin at 310K. Colonies from each of the

ThyX mutants were streaked onto LB agar plates with erythromycin and ampicillin and were grown overnight at 310K. Each of the different ThyX mutated strains was again streaked onto M9 minimal medium plates containing IPTG (200 µl of 100 mM IPTG added to the surface and allowed to dry) and either no thymidine or 50 µg/ml of thymidine. The plates were incubated at 310K after two days and were photographed.

4. FAD binding Assay

To test the FAD binding activity of wild-type or mutated ThyX, we over expressed and purified all the mutant proteins in the same way as wild-type ThyX. All these proteins were adjusted to the same concentration by BCA method and the absorbance at 450 nm were scanned.

RESULTS AND DISCUSSION

1. Overall Structure of *H. pylori* ThyX and Comparison with Homologous Structures

H. pylori ThyX forms a homo-tetramer with a diamond-like structure with D2 point-group (Fig. **1a**). A large catalytic pocket is formed in the center of the tetramer of *H. pylori* ThyX. Four FAD and four dUMP molecules, one each per monomer, are located at the active site near β1 and α5 with excellent electronic density (Fig. **1b**). A cleft with a total surface area of 8600 $Å^2$ is buried between these monomers. The overall structure of *H. pylori* ThyX monomer can be divided into three domains: a central α/β domain, a bottom helical domain and a top helical domain (Fig. **1c**). The overall folding pattern of *H. pylori* ThyX is nearly identical to those of homology structures such as *T. maritime, M. tuberculosis* and *PBCV-1* ThyX [13, 19, 28] and can be super-

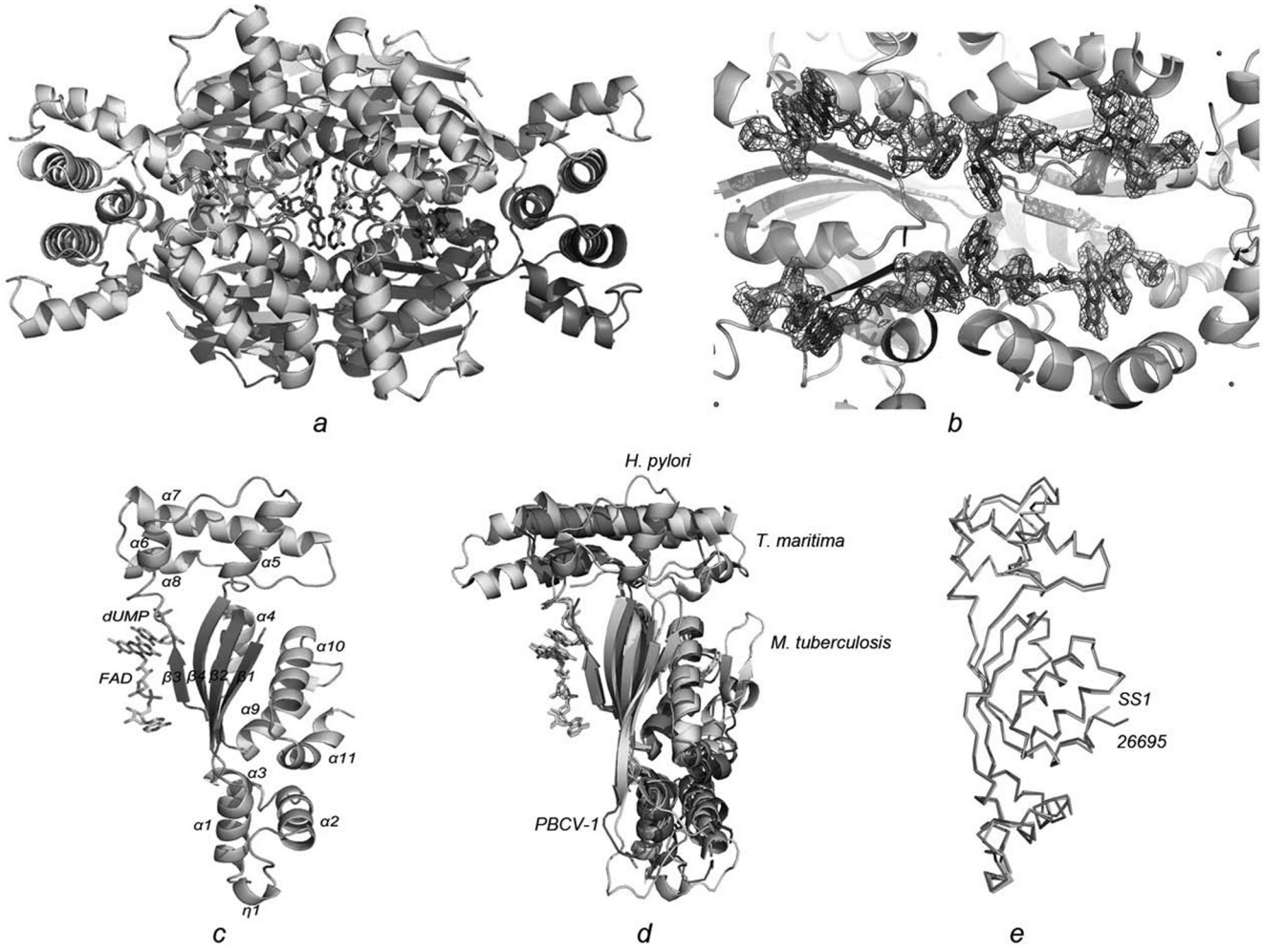

Figure 1. Overall structure of *H. pylori* ThyX. **a**) The top view of the ThyX homotetramer; the four monomers are colored differently. The co-factor FAD and substrate dUMP are shown as sticks. **b**) The FAD and dUMP binding sites; the electronic density of both moieties is of excellent quality. **c**) The monomer structure of the ThyX subunit B consists of 11 α-helices, 4 β-sheets and a 3^{10} helix, FAD and dUMP are located on the side of α5 and β1. **d**) Structural superposition of *H. pylori* ThyX with homologous structures from, *M. tuberculosis*, *T. maritima* and *PBCV-1*. Differences are mainly observed in the top helical domain. e) Structural superposition of *H. pylori* ThyX form stain 26695 and SS1.

posed with r.m.s.d of 1.852 Å, 1.812 Å and 1.978 Å, respectively, for all Cα atoms. *H. pylori* ThyX is unique in the top helical domain; helix α7 is much shorter than in other structures, and α5 and α6 are not observed in these structures (Fig. **1c**). Compared with the structure of ThyX from *H. pylori* strain SS1, the main difference also exists in the top helical domain, as shown in (Fig. **1e**), the α5 helix and the loop between α5 and α6 helix were slighted shifted by 10 degrees, where as the other parts resemble each other well.

2. The FAD and UMP Binding Sites

The purified *H. pylori* ThyX exhibits a characteristic yellow color, which indicates that it was in complex with oxidized FAD during purification, and the binding was verified by detecting the absorbance peak at 450 nm in its UV spectra [29]. Surprisingly, the three oxygen atoms from the ribityl group of the FAD, termed O2', O3' and O4', respectively, did not interact with residues of ThyX directly. In contrast, they were hydrogen bonded by the water molecules Tip15 and Tip 57; the two water molecules also formed hydrogen bonds with the N1 atom of FAD and the N atom of His71, respectively (Fig. **2a**) After superposing of the FAD in *H. pylori* ThyX with homologous structure, we believe that the difference may be attributing to the different conformation of the ribityl group, which may represent an intermediate state of the co-factor during the catalytic reaction.

Interestingly, dUMP was found in *H. pylori* ThyX structure even though no exogenous dUMP was added during all the purification and crystallization processes, which was also observed by Wang *et al* [18] but was not observed in homologous structures such as *M. tuberculosis*, *T. maritima* and *PBCV-1* ThyX [13, 19, 28]. Our results indicate that dUMP bound *H. pylori* ThyX with a higher affinity than homologous enzymes. The substrate dUMP was completely buried inside *H. pylori* ThyX and was mainly hydrogen bonded by several highly conserved residues, including Ser107, Ser108, Arg109, and Arg89 from subunit B and Glu94, Arg97, Arg197 from subunit D (Fig. **2b**). Quite surprisingly, the structural elements in *H. pylori* ThyX that involved in dUMP binding were almost identical to those in *T. maritima* ThyX, which was in complex with a dUMP analogue, BrdUMP, only the length of hydroid bonds vary between these two structures (supplementary Fig. **1**).

3. Sequence Alignment with Homologous Enzymes

The sequence of *H. pylori* ThyX was aligned with homologous enzymes from *M. tuberculosis*, *T. maritima*, *PBCV-1* ThyX and ThyX from *H. pylori* Srain SS1[13, 19, 28,18]. As shown in (Fig. **3**), although these enzymes share the same fold pattern, the identity of amino acid sequence is lower than 30% between each other. Among the 10 residues involved in FAD binding, 6 of which including His71, Glu76, Arg97, His98, Arg99 and Arg188 were completely conserved. Similar result was observed in the dUMP binding site. These results indicting that the active site of *H. pylori* ThyX was highly conserved among homologous enzymes.

As confirmed by sequence alignment, Arg70 is an insertion in *H. pylori* ThyX and is not observed in homologous enzymes from *M. tuberculosis*, *T. maritima* and *PBCV-1*

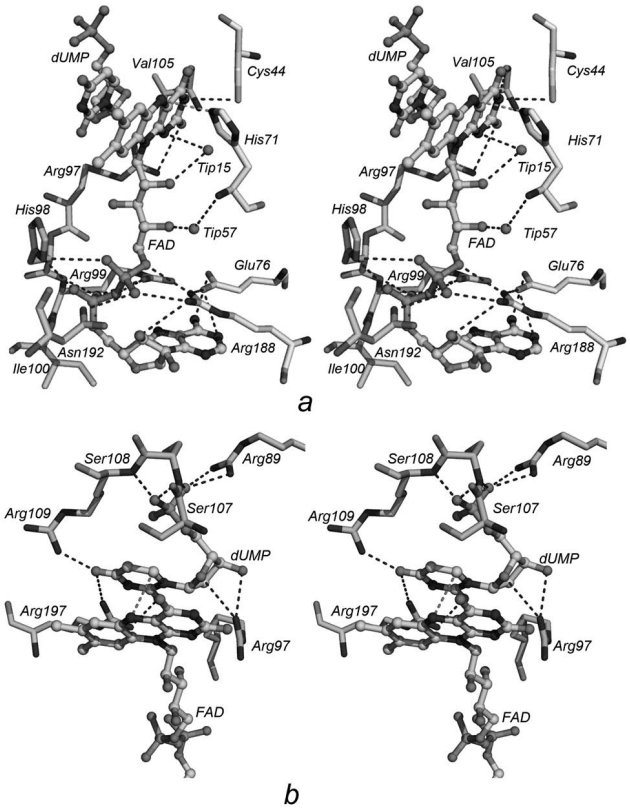

Figure 2. Detailed interactions between FAD, dUMP and residues from *H. pylori* ThyX. (**a**) The FAD binding site. **b**) The dUMP binding site. Dashes indicate putative hydrogen bonding interactions.

ThyX [13, 19, 28], the structures of which were available in the PDB.Since this residue is located adjacent to the cofactor FAD (Fig. **4**), and it has been reported that the oxidation activity of NADPH by *H. pylori* ThyX is much lower than the high specific activity of the PBCV-1 ThyX protein [30], we propose that the long side chain of Arg70 may interact with FAD during the catalytic process and resulting in the low activity of *H. pylori* ThyX.

4. Complementation Assay

Four mutants including H98K, R109A, R197A and S107A failed to complement the growth of the strain. S108A was able to functionally partially complement the growth of the auxotroph *E. coli*. As all these residues were directly involved in FAD and dUMP binding, the results could be explained that mutation of these residues may block the binding of these chemicals to the active site of ThyX or these chemicals were not stable as in wild type of ThyX, thus resulting in loss of enzymatic activity. Among these residues, four of which were completely conserved (Fig. **3**), only Ser108 was replaced by Gly and Glu in other species, this was in consistence with the result of complementation assay that mutant of this residue resulting in slight loss of enzymatic activity of *H. pylori* ThyX.

Figure 3. Sequences alignment of *H. pylori* ThyX with homologous enzymes. Colored outlines indicate identical and similar amino acid residues, respectively. The black asterisks indicate the amino acids mutated in this study.

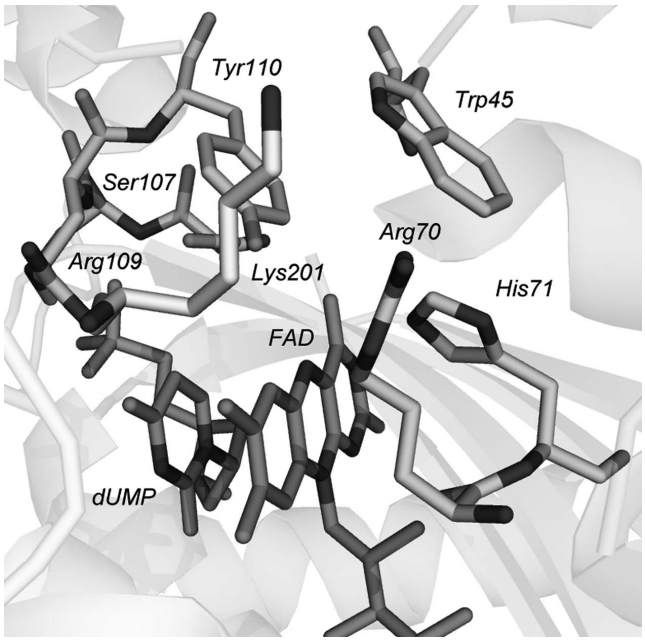

Figure 4. An insertion of residue Arg70 may responsible for the low enzymatic activity of *H. pylori* ThyX.

5. FAD binding Assay

The H98K mutant had lost most of its FAD binding ability, which could be explained by the important role played by this residue in FAD binding. The R109A mutant had lost approximately half of its FAD binding ability despite the fact that it is not involved in directly interact with FAD, suggesting that it plays a dual rule in both dUMP and FAD binding. Similarly to R109A, mutation of R197A retains half of the FAD binding ability but shows no complementary activity. The fact that the other mutants had no impact on FAD binding activity can be explained because these residues are far away from FAD and have no direct interaction with it.

In conclusion, we have determined the crystal structure of *H. pylori* ThyX in complex with substrate dUMP and co-factor FAD. Based on the structure, we indentified the key residues that are involved in co-factor and substrate binding. The importance of these residues was confirmed by genetic complementation and FAD binding assay by using ThyX mutants. An insertion of Arg70 may responsible for the low enzymatic activity of *H. pylori* ThyX as compared with homologous enzymes. These findings will facility the structure-based inhibitor design, and our screening for potential *H. pylori* ThyX inhibitors is currently in progress.

PDB ACCESSION CODE

The coordinates and structure factors have been deposited with the RCSB, Protein Data Bank with the accession code ID 3AH5.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publishers Web site along with the published article.

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