Crystal structure and enzymatic characterization of thymidylate synthase X from *Helicobacter pylori* strain SS1

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Abstract: Thymidylate synthase X (ThyX) catalyzes the methylation of dUMP to form dTMP in bacterial life cycle and is regarded as a promising target for antibiotics discovery. *Helicobacter pylori* is a human pathogen associated with a number of human diseases. Here, we cloned and purified the ThyX enzyme from *H. pylori* SS1 strain (HpThyX). The recombinant HpThyX was discovered to exhibit the maximum activity at pH 8.5, and K_m values of the two substrates dUMP and CH₂H₄folate were determined to be $15.3 \pm 1.25 \mu\text{M}$ and $0.35 \pm 0.18 \text{ mM}$, respectively. The analyzed crystal structure of HpThyX with the cofactor FAD and the substrate dUMP (at 2.31 Å) revealed that the enzyme was a tetramer bound to four dUMP and four FAD molecules. Different from the catalytic feature of the classical thymidylate synthase (ThyA), N5 atom of the FAD functioned as a nucleophile in the catalytic reaction instead of Ser84 and Ser85 residues. Our current work is expected to help better understand the structural and enzymatic features of HpThyX thus further providing valuable information for anti-*H. pylori* inhibitor discovery.

Keywords: thymidylate synthase; *Helicobacter pylori*; thymidylate synthase ThyA

Abbreviations: TS, thymidylate synthase; ThyX, thymidylate synthase X; ThyA, thymidylate synthase A; HpThyX, ThyX enzyme from *H. pylori*; TmThyX, ThyX from *Thermotoga maritima*; MtbThyX, ThyX from *Mycobacterium tuberculosis*; PBCV-1 ThyX, ThyX from *Paramecium bursaria* Chlorella virus-1; CH₂H₄folate, methylenetetrahydrofolate; H₄folate, tetrafolate.

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Introduction

Helicobacter pylori is a human pathogen associated with a number of human diseases including gastritis, peptic ulceration, and gastric cancer, although its rapid promulgation and infection have made this kind of pathogen a severe threat to human health.^{1–4} Generally, treatment of *H. pylori* infection could be performed by taking combinations of one proton pump inhibitor with two or three antibiotics.⁵ However, overuse and misuse of antibacterial agents have resulted in the emergence of antibiotic resistant strains for mutations on drug targets, making the multiple therapy regimens potentially ineffective in clinical use.⁶ Therefore, it has become urgent to discover novel anti-*H. pylori* agents acting new drug targets.

As an essential precursor for DNA synthesis, thymidylate is important for life maintenance. It has been discovered that the *de novo* synthesis of thymidylate in *H. pylori* is catalyzed by a newly identified

thymidylate synthase X (ThyX) (EC 2.1.1.148) instead of the classical thymidylate synthase A (ThyA) (EC 2.1.1.45).^{7,8} Using methylenetetrahydrofolate ($\text{CH}_2\text{H}_4\text{folate}$) as one-carbon donor and reduced-flavin nucleotides as reductive equivalents, ThyX catalyzes dUMP into dTMP with tetrafolate (H_4folate) as product. Enzymatic assays combining gel filtration revealed that active ThyX behaved as a homo-tetramer.^{7,9} Crystal structure analyses of TmThyX (ThyX from *Thermotoga maritima*), MtbThyX (ThyX from *Mycobacterium tuberculosis*), and PBCV-1 ThyX (ThyX from *Paramecium bursaria* Chlorella virus-1) demonstrated that ThyX structure exhibited no significant similarity to any other protein structures, and its tetramer consisted of four identical monomers bound with four FAD molecules.^{9–12} Although ThyX and ThyA both catalyze the *de novo* synthesis of thymidylate, they exhibit different structural and catalytic features between each other. For example, the putative active pocket of *H. pylori* ThyX (HpThyX) lacks the cysteine residue, which is conserved and essential for ThyA activity.^{13,14} Thus, HpThyX has been regarded as a promising drug target for anti-*H. pylori* agent design.

Recently, several ThyA inhibitors that are analogs of folate or dUMP, for example, monophosphate 5-fluoro-2'-deoxyuridine (5-FdUMP), *o*-propargyl-5,8-dideazafolate, 10-methylfolate, and 8-azapurinone deoxyribonucleoside 5'-phosphate, have been reported showing inhibition activities against ThyX^{7,15–20}; and also a few inhibitors that are not analogs of folate or dUMP were reported.²¹ As ThyA is the only thymidylate synthase contained in human body, novel nonsubstrate/cofactor analog inhibitors with specific activity against ThyX are thus well suited for anti-*H. pylori* agent exploration. However, despite the strong desire for such a kind of inhibitor development, the catalytic mechanism of ThyX is still debated. Until recently, most of the published models proposed that the reduction step within ThyX catalysis was carried out by a conserved serine residue as a nucleophile,^{10–12,15,18–20} whereas later ThyX catalysis was also suggested to be actually independent on such a nucleophile but subject to the reduced FADH_2 by directly transferring a hydrogen to dUMP.²² Therefore, elucidation of HpThyX structure has become much valuable for better understanding ThyX family catalytic mechanism.

In this work, we enzymatically characterized HpThyX enzyme and determined its crystal structure complexed with the cofactor FAD and substrate dUMP. The results revealed that the enzyme was a tetramer with four identical subunits harboring four FAD and four dUMP molecules. By comparing HpThyX with the two reported bacterial ThyX (MtbThyX and TmThyX) in structure,^{10,11} the resi-

dues recognizing dUMP and FAD were determined to be not completely conserved except those binding both the substrate and the cofactor. Notably, different from the catalytic feature of classical thymidylate synthase (ThyA), rather than Ser84 or Ser85, N5 atom of FAD was suggested as a nucleophile to attack the carbon atom to be methylated (C6) on dUMP. Our work is expected to help better understand the structural features of HpThyX in the presence of its cofactor (FAD) and substrate (dUMP), and obtain useful information on the essential catalytic mechanism of HpThyX.

Results

Preparation of the recombinant ThyX enzyme

Using the genome of *H. pylori* strains J99 and 26695 as referenced templates for primers designing, the gene of *HpThyX* was successfully amplified by PCR from the genome of *H. pylori* strain SS1. Sequencing result showed that *HpThyX* gene was a 627 base-pair fragment including the stop codon. The amplified fragment was constructed into vector pQE30 to generate the expression plasmid *pQE30-HpThyX*. HpThyX enzyme was thus expressed in large amount in *Escherichia coli* strain M15 as a His-tagged fusion protein. The recombinant proteins expressed mostly as soluble forms in bacterial extracts. Usually, 20–40 mg of purified HpThyX (1 L culture medium) could be yielded by nickel chromatography followed by size exclusion chromatography. The purified HpThyX solution was bright yellow, indicative of the possible binding of flavin molecule of FAD, which has been confirmed in later crystal structure analysis. As shown in SDS-PAGE result [Fig. 1(A)], HpThyX migrated as a single band (Lane 1) at 25 kDa according to the standard protein markers (Lane 2). Mass spectral analysis further figured out the molecular mass of the recombinant His-tagged HpThyX protein as 25,066.0 Da [Fig. 1(B)], consistent with the molecular mass (25,019.55 Da) calculated from its amino acid sequence.

Enzymatic assay

To scrutinize the enzymatic kinetics of the recombinant HpThyX protein, dUMP labeled with ^3H was used as substrate. As shown in Scheme 1,^{7,22} the reaction started under the catalysis of the recombinant HpThyX, and the loss of ^3H of [5- ^3H] dUMP into H_2O during the formation of dTMP was detected by Beckman LC6500 liquid scintillation counter (data not shown), which indicated that the recombinant HpThyX exhibited thymidylate synthase activity. To determine the kinetic parameters of HpThyX, the amount of ^3H in the generated H_2O was measured at different concentrations of dUMP or $\text{CH}_2\text{H}_4\text{folate}$. The reciprocal of the calculated velocity of the catalytic reaction was plotted against

Figure 1. Purity (A) and mass spectral identification (B) of the recombinant HpThyX. Purified HpThyX ran as a single band on 10% SDS-PAGE (A). Left lane, purified ThyX protein. Right lane, molecular weight standard marker. Molecular mass in kDa is indicated on the right.

the reciprocal of the molar concentration of dUMP, and then, the apparent K_m value for dUMP was evaluated as $15.3 \pm 1.25 \mu M$ by Michaelis–Menten equation [Fig. 2(A)]. Furthermore, the apparent K_m value for CH_2H_4 folate was also worked out as $0.32 \pm 0.18 mM$ according to the similar method [Fig. 2(B)]. On the other hand, the contribution of NADPH to the catalytic reaction was characterized by changing its concentrations in standard reaction mixture. Interestingly, $100 \mu M$ of NADPH could saturate the enzymatic reaction, although excess NADPH decreased HpThyX activity instead [Fig. 2(C)].

Catalytic reaction assays at different pHs indicated that HpThyX showed an increase of activity from pH 6.5 to 8.5 but a decrease from pH 8.5 to

10.5, with the highest activity around pH 8.5. [Fig. 2(D)]

Overall structure

The crystal structure of HpThyX was refined to R_{work} and R_{free} factors of 17.4% and 22.3%, respectively. All loops were observed in the final crystallographic model. As shown in Figure 3(A,B), HpThyX structure was a classical tetramer with four identical subunits forming extensive interactions, similar to the cases for MtbThyX, TmThyX, and PBCV-1 ThyX,^{10–12} where four FAD molecules and four dUMP molecules were observed in HpThyX tetramer active sites. Each active site was formed by residues from three neighboring subunits. Furthermore, one

Scheme 1. The equation of ThyX catalyzed reaction. ThyX enzyme activity was measured by detecting the amount of 3H in the generated H_2O , the substrate dUMP was labeled with 3H , $R = 2'$ -deoxyribose-5'-phosphate, $R' = p$ -aminobenzoyl-glutamate.

Figure 2. Enzymatic kinetics characterization of recombinant HpThyX. Kinetic analyses were performed for (A) dUMP, (B) $\text{CH}_2\text{H}_4\text{folate}$, and (C) NADPH. Enzymatic activities of HpThyX were determined at different concentrations of the indicated substrates and cofactors, with all other substrates at fixed saturated concentrations as described in "Experimental Procedures" Section. (D) The enzymatic activities of recombinant HpThyX at different pH values. The assays were carried out in standard reaction mixture at a pH range from 6.5 to 10.5. The recombinant HpThyX showed its maximal activity at pH 8.5, and then, the activity of the enzyme at pH 8.5 was taken as 1.0.

additional dUMP molecule lies on the crystal contact interface, possibly for facilitating the crystal growth.

As indicated in the overall monomer structure [Fig. 3(B)], each monomer of HpThyX contained a top helical domain, a central α/β domain, and a bottom helical domain. The top helical domain was constituted by a 20-residue long helix ($\alpha 6$) and three short helices ($\alpha 4$, $\alpha 5$, and $\alpha 7$). Two loops connected the top helical domain to the $\beta 3$ and $\beta 4$ strands of the central domain. The central domain contained a four-antiparallel β sheet with strands $\beta 1$ – $\beta 4$. These sheets were curved and covered several helices on the other side [$\alpha 3$, connecting $\beta 2$ and $\beta 3$, and C-terminal helices $\alpha 8$, $\alpha 9$, $\eta 3$, $\eta 4$ (η stands for 3_{10} helix)], forming the core of the subunit. The bottom helical domain was composed of helices $\alpha 1$, $\alpha 2$, $\eta 1$, and $\eta 2$. Similarly, two loops linked the bottom helical domain to the central domain through $\beta 1$ and $\beta 2$.

The total surface area of the tetramer buried between the different subunits was more than $6000 \text{ \AA}^2$. The largest surface area was buried between subunits A and B, which was $1779 \text{ \AA}^2$ per monomer, and the smallest surface area buried between subunits A and C was $384 \text{ \AA}^2$ per monomer.

Cofactor binding site

As demonstrated in protein preparation section, the purified HpThyX enzyme was bright yellow, implying the possibility that the oxidized form of the cofactor FAD remained in the protein. Crystal structure analysis confirmed this hypothesis. Similar to the other published structures of ThyX enzymes, like MtbThyX, TmThyX, and PBCV-1 ThyX,^{10–12} HpThyX tetramer contained four FAD molecules [Fig. 3(A)]. As all four active sites in a tetramer were essentially identical, we described here only one active site composed by subunits A, B, and D as an example.

As indicated in Figure 4(A), FAD interacted directly with these subunits through 20 hydrogen bonds (the atoms of FAD interacting with these residues were labeled in Supporting Information, Fig. S1A). Val82 from subunit A formed two hydrogen bonds with O2 oxygen and N3 nitrogen atoms of the flavin ring from FAD.

Four residues (Arg74, Arg76, Asn169, and Arg174) from subunit B made 12 hydrogen bonds. NH1 and NH2 nitrogen atoms of Arg74 both contacted with O2 oxygen of FAD, and NH2 nitrogen

Figure 3. Overall structure of HpThyX. (A) Tetramer structure. Subunits A, B, C, and D are colored green, cyan, yellow, and magenta, respectively. (B) Monomer structure. Superimposition of chains A, B, C, and D. The color scheme is the same as that of Figure 2A.

also interacted with O3' oxygen atom of ribityl group in FAD. The main-chain of Arg74 interacted with O1A oxygen atom from the phosphate group of the AMP in FAD. Arg74 interacted with two water molecules W220 and W241, whereas W241 interacted with O3' oxygen atom of FAD. Arg76 interacted with FAD through five hydrogen bonds. The main-chain of Arg76 contacted with O1A and O2A oxygen atoms of FAD, and O1A oxygen also interacted with NE and NH2 nitrogen atoms of Arg76. Moreover, this NH2 nitrogen atom made the fifth H-bond interaction with O1P oxygen from the phosphate group of the FMN in FAD. Asn169 formed two hydrogen bonds with O2P oxygen and O3P oxygen of FAD. Additionally, Asn169 bound to water molecule W212, which also interacted with O3P and O2A oxygen atoms of FAD. Arg174 formed a hydrogen bond with O4' oxygen atom from the ribityl group of FAD.

Four residues (Arg47, Ser50, Glu53, and Arg165) from subunit D interacted with FAD through six

hydrogen bonds. The main-chain of Arg47 contacted with O2' oxygen atom from the ribityl group of FAD. Moreover, the main-chain of Arg47 and O2' oxygen both made hydrogen bonds with water molecule W217. The side-chain of Ser50 formed a hydrogen bond with O2' oxygen atom of FAD. Additionally, the side-chain and main-chain of Ser50 both interacted with water molecule W217. OE1 oxygen atom of Glu53 made one hydrogen bond with N6A nitrogen of the ribose sugar of FAD. NH1 nitrogen atom of Arg165 formed one H-bond with O1P oxygen, whereas NH2 of Arg165 formed two hydrogen bonds with O2P and O3B of FAD. In HpThyX-FAD-dUMP complex structure, the flavin ring of FAD was exposed to the surface of the tetramer and stacked to the pyrimidine ring of the substrate dUMP. The distance between N5 of the flavin ring and C6 of dUMP was 3.37 Å (Fig. 5), which is suitable for a hydrogen atom to be transferred to dUMP. The ribityl and AMP parts of FAD sat deeply into the protein, stabilizing FAD for exerting its function.

Figure 4. Active site structure of HpThyX. The cofactor-binding site of HpThyX composed of subunits A, B, and D, which colored green, cyan, and magenta, respectively, and hydrogen bonds are presented as dotted lines (same below). The substrate-binding site of HpThyX formed by residues from subunits A and B, the color scheme is the same as that of Figure 3A.

Substrate-binding site

As described above, three subunits in the catalytic center contacted with the cofactor FAD, whereas two

subunits associated with each substrate dUMP through 14 hydrogen bonds [Fig. 4(B)]. In the case of subunits A and B, the hydrogen bonds involved

Figure 5. Key active sites in HpThyX reaction. The side-chains of Ser84 and Ser85 are 4.13 and 7.04 Å away from C6 of dUMP, respectively. N5 of FAD is only 3.37 Å away from C6 of dUMP. Hydrogen bonds are presented as dotted lines in red.

five residues from chain A (Arg66, Ser84, Ser85, Arg86, and Lys147), and three residues from chain B (Glu71, Arg74, and Arg174). The pattern of dUMP interacting with these residues was labeled in Supporting Information, Figure S1B.

In subunit A, NH1 and NH2 nitrogen atoms of Arg66 interacted with OP1 oxygen atom of dUMP through two hydrogen bonds. Furthermore, NH2 nitrogen of Arg66 and OP1 oxygen of dUMP both interacted with water molecule W214. The side-chain of Ser84 made a hydrogen bond with OP2 oxygen atom of dUMP. The main-chain of Ser85 interacted with OP1 and OP2 of dUMP through two hydrogen bonds, and this OP1 also formed a hydrogen bond with the side-chain Ser85. The main-chain nitrogen atom of Arg86 contacted with OP2 oxygen atom of dUMP, and NH1 nitrogen atom of this arginine interacted with O4 of dUMP. The side-chain nitrogen of Lys147 formed hydrogen bond with OP3 of dUMP, and these two atoms both interacted with water molecule W214.

Three residues from subunit B interacted directly with dUMP through five hydrogen bonds. Glu71 made two hydrogen bonds with OP3 and O4 of dUMP. Moreover, Glu71 and O4 also interacted with water molecule W216. NH1 of Arg74 contacted O3' of dUMP through a hydrogen bond. NH1 and NH2 of Arg174 both interacted with O2. Furthermore, NH1 of this arginine and O4 both interacted with water molecule W216.

Additionally, it was discovered that the conserved residues Arg74 and Arg174 made close contacts with both cofactor FAD and the substrate dUMP [Figs. 4(A,B) and 6], implying that these two residues might play important roles in enzymatic reaction.

Structure comparison with other *ThyX* enzymes and flavoenzyme

Generally, the structure of HpThyX was similar to those of the bacterial *ThyX* enzymes TmThyX and MtbThyX (Supporting Information, Fig. S2). The r.m.s. deviation between TmThyX and HpThyX was 2.2 Å, and their sequences shared 29% identity, whereas the r.m.s. deviation was 1.87 Å between MtbThyX and HpThyX, and their sequence identity was 25%. The angle between the mass center of domain and the hinge residue connected with the next domain was discussed for evaluation of the domain orientation among the three structures (HpThyX, TmThyX, and MtbThyX). It was determined that the angles between domain 1 (top helical domain) and domain 2 (central α/β domain) were 74.5°, 73.4°, and 76.2° in HpThyX, TmThyX, and MtbThyX, respectively, whereas the angles of domain 2–domain 3 (bottom helical domain) were correspondingly 130.6°, 124.8°, and 132.8°. These results thus indicated that the differences in mutual orientation of

the domains were mainly in small variations of the bottom helical domain, whereas other domain orientations were similar to each other in all three *ThyX* structures. Moreover, cofactor FAD and the substrate dUMP located similarly in the three different *ThyX* structures (Supporting Information, Fig. S2). By superposing the key FAD binding residues (indicated in the sequence alignment, Fig. 6), FAD molecule of HpThyX had an r.m.s. deviation of 0.99 Å compared with that of TmThyX, and 0.32 Å to MtbThyX; calculating similarly for UMP molecules, an r.m.s. deviation of 0.47 Å was determined between HpThyX and TmThyX, and 0.38 Å between HpThyX and MtbThyX. These values indicated that the binding patterns of FAD and dUMP were very similar in all three *ThyX* enzymes.

As shown in Figure 6, the completely conserved residues were shaded in red. Among them, some formed hydrophobic core of the protein (Val3, Phe59, Phe202, Ala179, Thr156, and Pro151), and some located on the subunit interface (His54, Ala17, Ser79, Asn163, Tyr87, Ala179, and Pro151), which were presumably involved in stabilizing the tetramer structure. Notably, several completely conserved residues bound cofactor FAD and substrate dUMP in the active site. For example, of the five arginine residues forming hydrogen bonds with FAD [Fig. 4(A)], four (Arg74, Arg76, Arg165, and Arg174) are completely conserved (Fig. 6). Among them, Arg74 and Arg76 contributed to the binding of FAD using both main-chain and side-chain atoms. These completely conserved and positively charged residues were suggested to stabilize the anionic form of reduced FADH₂,¹² thus critical for catalysis. As for the substrate binding, four of the eight dUMP-binding residues were completely conserved (Arg74, Ser84, Arg86, and Arg174, Fig. 6), forming six hydrogen bonds with the substrate [Fig. 4(B)].

However, the structure-based sequence alignment in Figure 6 revealed that the substrate and cofactor binding residues of these enzymes were not completely conserved (e.g., Arg47, Val82, and Lys147) except those recognizing both dUMP and FAD. It was found that the nonconserved Ser85 of HpThyX formed hydrogen bonds with the phosphate group of substrate dUMP by its side-chain. The conserved residue Ser84 accompanying Ser85 “clamped” one side of the dUMP phosphate group (Fig. 5), implying the key potential roles for these two serine residues in substrate binding, in accordance with the observation that Ser84/Ser85 double mutation could deactivate HpThyX.¹⁶ Notably, because neither of these two serine residues was within the H-bond range to C6 of dUMP (the carbon to be methylated), they were unlikely to act as nucleophiles to attack C6-dUMP during catalysis.

As indicated in Figure 7, the distance between C6 of dUMP and N5 of FAD in HpThyX, TmThyX, and MtbThyX structures was around 3.5 Å, which

Figure 6. Structure-based sequence alignment of HpThyX, TmThyX, and MtbThyX. PDB codes are indicated beside the protein names. Secondary structures of HpThyX are labeled. Residues involved in the binding network are labeled, and the alignment was produced by the program ESPript.²³

thus made it possible for the nucleophilic attack between these two atoms (C6 and N5), further implying the potential catalytic mechanism for ThyX using N5 atom of FAD as nucleophile to attack C6 atom of dUMP. And this catalytic mechanism was already well established in other flavoenzyme, like uridine 5'-diphosphate galactopyranose mutase (UGM or Glf), which catalyzed the interconversion of UDP-galactopyranose and UDP-galactofuranose. Crystal structure of UGM from *M. tuberculosis* (MtbUGM) showed that N5 atom of FAD acted as a nucleophile to attack C1 atom of the substrate, an essential step for catalysis, and the distance between these two atoms was about 3.6 Å (Fig. 7).²⁴ Considering that the uracil ring of the substrate was almost perfectly parallel to the riboflavin ring of FAD in all three ThyX struc-

tures, we used the angle C5(dUMP)–C6(dUMP)–N5(FAD) to evaluate the angular relationship between N5 of FAD and C6 of dUMP. In all three ThyX structures, this angle was around 70° (Fig. 7). Therefore, our results indicated the similar positioned status for either of N5 or C6 in the known ThyX structures. Additionally, MtbUGM structure demonstrated that the counterpart angle C2(substrate)–C1(substrate)–N5(FAD) was 74.0° (Fig. 7), similar to that in ThyX structures.

Discussion

ThyX is a newly discovered family of thymidylate synthase.¹⁵ Compared with the classical ThyA, ThyX exhibits different structure and reductive mechanisms.^{8–10,15–17,25} Structure and enzymatic kinetics

Figure 7. Structures of flavoenzyme-FAD-substrate complex. *HpThyX* (PDB:3N3Y), *TmThyX* (PDB:1O26), *MtbThyX* (PDB:2AF6), and *MtbUGM* (PDB:3INT). The distances between N5 of FAD and C6/C1 of substrate are 3.37 Å, 3.48 Å, 3.35 Å, and 3.57 Å for *HpThyX*, *TmThyX*, *MtbThyX*, and *MtbUGM*, respectively, whereas the corresponding angles C5/C2(substrate)-C6/C1(substrate)-N5(FAD) are 75.4°, 78.0°, 69.7° and 74.0°.

investigation in our work has revealed a series of interesting findings. For *HpThyX*, its K_m ($15.3 \pm 1.25 \mu M$) against dUMP was similar to that of *P. bursaria* Chlorella virus-1 ($15 \mu M$),²⁰ although a little higher than that of *ThyX* from *Chlamydia trachomatis* ($5.99 \pm 0.54 \mu M$).¹⁵ Similar to *TmThyX*¹¹ and *MtbThyX*,¹⁰ *HpThyX* tetramer contained four

cofactor/substrate binding sites, and each monomer involved three domains. The active site residues were not completely conserved among different *ThyX* enzymes except those recognizing both substrate and cofactor. It was noted that in *HpThyX*, there were nonconserved residues (e.g., Ser85) interacting with the substrate dUMP, implying their potent roles in substrate binding.

Currently, three types of catalytic mechanisms for *ThyX* have been proposed. The first was the so-called ping-pong mechanism for *MtbThyX*,¹⁰ *TmThyX*,^{11,19} and *CtThyX*¹⁵; the second was the sequential mechanism for PBCV-1 *ThyX*^{12,20} and L5/D29 *ThyX*.²⁶ These two mechanisms shared an essential characteristic that in the active site of the enzyme, one highly conserved serine residue as a catalytic nucleophile attacked the C6 of the substrate dUMP, thereby facilitating the cofactor FADH₂ to transfer hydrogen to dUMP. The third type of the catalytic mechanism was recently proposed by Kohen and coworkers,²² in which the *ThyX*-catalyzed reaction did not depend on serine as catalytic nucleophile, rather directly on the reduced FADH₂, which also provided hydrogen to C6 of dUMP.

As indicated in the structure of *HpThyX*, the distance between N5 of FAD and C6 of dUMP was 3.37 Å, indicating that FADH₂ could easily transfer one hydrogen to C6 of dUMP. Similar to those of *TmThyX*, *MtbThyX*, and PBCV-1 *ThyX*,^{10–12} the conserved Ser84 of *HpThyX* was around 4.00 Å away from C6 of dUMP, whereas the nonconserved Ser85 was 7.04 Å from C6-dUMP (Fig. 5). According to the published result,²² if a serine residue was to serve as a nucleophile in *ThyX* catalytic reaction, it must be activated by a basic environment in the active site of the enzyme. However, we did not observe such a basic system that could activate the serine residue in *HpThyX* structure. Additionally, the hydroxyls of Ser84 and Ser85 were passivated by hydrogen bond formation with dUMP phosphate group (Fig. 5), making them even more unlikely to act as nucleophiles to attack C6-dUMP. Thus, our determined crystal structure of *HpThyX* indicated that neither Ser84 nor Ser85 could serve as catalytic nucleophile in *HpThyX* enzymatic reaction. Nevertheless, Ser84 and Ser85 still played pivotal roles in substrate binding for *HpThyX*, as demonstrated in Figure 5, where Ser84 and Ser85 “clamped” the substrate dUMP through hydrogen bonds to stabilize the substrate at a favorable position for C6-dUMP easily accepting the hydrogen from the reduced FADH₂. This is in accordance with the observation that double mutations (S84A/S85A) deactivated the enzyme.¹⁶

In summary, we characterized the crystal structure and enzymatic properties of *ThyX* from *H. pylori* strain SS1 (*HpThyX*). The structure of *HpThyX* complexed with the cofactor FAD, and the

substrate dUMP revealed conserved structural features of ThyX enzyme family, and showed that either Ser84 or Ser85 failed to serve as catalytic nucleophiles, different from the catalytic feature of ThyA. HpThyX performed catalysis relying on the reduced FADH₂, whose hydrogen was supposed to transfer directly to dUMP. Our current work is expected to help better understand the structural and enzymatic features of ThyX, thus providing useful information for anti-*H. pylori* inhibitor discovery targeting this enzyme.

Experimental Procedures

Materials and strains

(6R,S)-5,10-Methylene-5,6,7,8-tetrahydrofolic acid (CH₂H₄folate) was purchased from Schircks (Switzerland). [5-³H]dUMP was from Moravsek Biochemicals (CA). The genome DNA extract kit was purchased from Sangon (China). Crystal screen kits were purchased from HAMPTON and QIAGEN. *H. pylori* strain SS1, *E. coli* strains DH5 α , BL21 (DE3), and M15 are routinely maintained in our laboratory. All chemicals were of reagent grade or ultrapure quality, and commercially available.

Cloning of ThyX gene from *H. pylori* strain SS1

The genomic DNA of *H. pylori* strain SS1 was prepared using the Genomic DNA Extraction Kit (Sangon, China) and quantified by agarose gel electrophoresis. Based on the sequences of *H. pylori* strains 26695 and J99 (GenBank Accession Nos. NC_000915 and NC_000921), two primers were used to amplify the corresponding region including *thyX* gene on the chromosome of *H. pylori* strain SS1. The forward primer was: 5'-CTTAGAAATCGCTATGCGTTTGAA-3' and the backward primer was: 5'-CCTACAACAAACCCCT-TAAAA-3'. The PCR reaction was performed for 30 cycles: 45 s at 94°C, 45 s at 51°C, and 45 s at 72°C. The amplified DNA segment was purified and sequenced. Then another pair of primers (sense: 5'-AAAGGATC CATGGAAGTGATTTGTAAG-3' and antisense: 5'-TTT CCTAGGCTAATGCTTCAAACAATCTTC-3') were used to amplify the fragment. After being digested by *Bam*HI and *Hind*III restriction endonucleases, the PCR product was ligated into the expression vector pQE30 (QIAGEN) to produce a recombinant plasmid with the gene of *HpThyX* (pQE30-*HpThyX*). The nucleotide sequence of *thyX* gene from *H. pylori* strain SS1 has been deposited in the GenBank database (GenBank Accession No. AY601706).

Expression and purification of recombinant *HpThyX* and *Se-HpThyX*

His-tagged HpThyX—the constructed pQE30-*HpThyX* plasmid was transformed into *E. coli* strain M15. The cells were then inoculated into LB media

containing ampicillin (100 μ g/mL) and kanamycin (50 μ g/mL) at 37°C. When the OD₆₀₀ of the culture reached 0.6, the recombinant protein was induced by isopropyl- β -D-thiogalactopyranoside (IPTG) at a final concentration of 0.4 mM and cultured at 37°C overnight. Cells were harvested by centrifugation and suspended in 50 mL of Buffer A (5 mM imidazole, 500 mM NaCl, and 20 mM Tris-HCl, pH 7.4). After sonication on ice, cell lysates were clarified by centrifugation at 20,000g for 30 min at 4°C. The supernatant was added to 3 mL nickel metal chelating column followed by washing with 15 mL Buffer A, and then 15 mL Buffer B (40 mM imidazole, 500 mM NaCl, and 20 mM Tris-HCl, pH 8.0). Finally the recombinant protein was eluted from the column with Buffer C (250 mM imidazole, 500 mM NaCl, and 20 mM Tris-HCl, pH 8.0), and imidazole was removed by dialysis against Buffer D (50 mM Tris-HCl, pH 8.5). The dialyzed protein was concentrated using the Centrifugal filter devices (Millipore) and then centrifuged at 15,000g to remove the deposit. All the purification, dialysis and concentration procedures were performed at 4°C. Protein concentration was determined by Bradford assay using bovine serum albumin as standard.

HpThyX—for gaining better quality of *HpThyX* crystals, we produced a recombinant plasmid (pGEX6P-1-*HpThyX*) with *HpThyX* gene and the expression vector pGEX6P-1 (Amersham Pharmacia Biotech). The constructed pGEX6P-1-*HpThyX* plasmid was transformed into *E. coli* strain BL21 (DE3). Then, the cells were inoculated into LB media containing ampicillin (100 μ g/mL) at 37°C. When OD₆₀₀ of the culture reached 1.0, the recombinant protein was induced by IPTG at a final concentration of 0.4 mM and cultured at 18°C for additional 20 h. Cells were harvested by centrifugation and suspended in 50 mL of PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄, pH 7.5). After sonication on ice, cell lysates were clarified by centrifugation at 20,000g for 30 min at 4°C. The supernatant was loaded on a GSH-Sepharose column in PBS. The material was subjected to PreScission Protease digestion for 20 h. Finally, the cleaved GST protein was removed by a GSH-Sepharose column. The *HpThyX* was further purified by superdex 200 size exclusion chromatography in 20 mM Tris, pH 8.0, 200 mM NaCl, and 2 mM DTT. The purified protein was concentrated using the Centrifugal filter devices (Millipore) to 10 mg/mL.

Se-HpThyX—pGEX6P-1-*HpThyX* plasmid was used for selenomethionyl (Se-Met) protein (Se-*HpThyX*). The procedure for preparation of the Se-*HpThyX* was mainly according to the literature approach²⁷ in *E. coli* strain. Inoculation of 1 L M9 media with 50 mL overnight LB culture and grew until OD₆₀₀ = 1.0. Then, 100 mg threonine, lysine hydrochloride, and phenylalanine and 50 mg leucine,

Table I. Statistics of Diffraction Data and Structure Refinement

<i>Data collection statistics</i>	
Space group	<i>P</i> 2 ₁
Unit cell	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	65.53, 79.96, 96.41
α , β , γ (°)	90, 103.14, 90
Wavelength (Å)	0.9794
Resolution range (Å)	50–2.31
Completeness (%)	99.6 (98.4) ^a
<i>R</i> _{sym} (%)	6.3 (31.8)
$\langle I \rangle / \sigma$	28.0 (4.2)
Redundancy	3.7 (3.2)
<i>Refinement statistics</i>	
Asymmetric unit	Tetramer
Number of reflections	
Working set	42,533
Test set	2144
Number of atoms	
Protein	6796
dUMP	100
FAD	212
Water	147
Rmsd bonds (Å)	0.007
Rmsd angles (°)	1.1
<i>R</i> _{work} (%) ^b	17.4
<i>R</i> _{free} (%) ^c	22.3
<i>B</i> -factors (Å ²)	
Protein	44.3
dUMP	38.9
FAD	33.9
Water	38.4
<i>Ramachandran plot</i>	
Most favored (%)	97.6
Additionally allowed (%)	2.4
Disallowed (%)	0

^a Values for the outer shell are given in parentheses.

^b $R_{\text{work}} = \sum hkl | |F_{\text{obsd}}| - |F_{\text{calcd}}| | / \sum hkl |F_{\text{obsd}}|$, where F_{obsd} and F_{calcd} are observed and calculated structure factors, respectively.

^c For R_{free} , the above summation is extended over a subset of reflections (5%) that were excluded from all stages of refinement.

isoleucine, and valine was added to the culture, followed by the addition of 60 mg L-Se-Met, and 15 min later, the culture was induced with 1 mL of 1M IPTG (final concentration = 1 mM). The expression and purification of Se-HpThyX protein were similar to those of HpThyX.

Mass spectra-based identification of HpThyX

The LC/MS system for analyzing the mass spectra of His-tagged HpThyX combined HP1100 (Agilent) LC system with LCQ-DECA mass spectrometer (Thermo Finnigan) as previously described.²⁸ The purified enzyme (1.5 mg/mL) was injected to the column by an autosampler and separated at a flow rate of 0.2 mL/min. The scan mass range was from *m/z* 200 to 2000.

Enzymatic characterization of HpThyX

The enzymatic activity of HpThyX was assayed by measuring the loss of tritium of [5-³H] dUMP (spe-

cific activity 13.6 Ci/mmol) mainly according to the published methods.^{7,15–17,19,20} All the reactions were carried out at 37°C in nitrogen atmosphere to avoid oxidization of CH₂H₄folate. The optimized standard reaction mixture contained 50 mM Tris-HCl pH 8.0, 1 mM CH₂H₄folate, 10 mM MgCl₂, 2 mM NADPH, 0.5 mM FAD, HpThyX 225 µg/mL, and 0.55 µM [5-³H] dUMP in a total volume of 100 µL. The reaction was initiated by addition of purified HpThyX and terminated by 1 mL 10% active charcoal (m/v) in 2% TCA 10 min later. Then, the sample was mixed at room temperature for 1 h and centrifuged at 15,000g at 4°C for 15 min to remove the active Charcoal. The supernatant was added into the solution [1:1 (v/v) methanol and scintillation fluid] and gently mixed to get a clarified solution. Radioactivity in the supernatant was quantitated in a Beckman LC6500 liquid scintillation counter. The reaction mixture without enzyme was used as blank. In the *K_m* determination of CH₂H₄folate, the substrate concentration of CH₂H₄folate ranged from 0.1 to 3 mM with other substrates in saturation. During the measurement of the *K_m* value against dUMP, the concentrations of dUMP varied from 0.55 to 2.94 µM. For the enzymatic assays against NADPH, the concentration of NADPH was ranged from 0.1 to 4 mM. The *K_m* value of CH₂H₄folate was determined by directly fitting data to Michaelis–Menten equation [Eq. (1)]. The *K_m* value of dUMP was determined by fitting data to Lineweaver–Burk equation [Eq. (2)]. For all assays, the reaction mixture without enzyme was used as background, and the reactions were repeated three times for results confirmation.

$$v = \frac{V_m[S]}{[S] + K_m} \quad (1)$$

$$\frac{1}{v} = \frac{1}{V_m} + \frac{K_m}{V_m[S]} \quad (2)$$

v, velocity; *V_m*, the maximal enzyme velocity; *K_m*, the Michaelis–Menten constant; [S], the concentration of substrate.

Effects of pH on HpThyX activity

To investigate the possible pH effects on HpThyX enzymatic activity, optimized standard reaction was performed in Tris-HCl buffer within a wide pH range from pH 6.5 to 10.5. All the reactions were carried out in nitrogen atmosphere to avoid CH₂H₄folate oxidization. All the reactions were repeated three times to confirm the results.

Crystallization

The purified HpThyX (10 mg/mL) was incubated with 4 mM dUMP on ice overnight, and 1 µL of the complex was then mixed with 1 µL of the reservoir solution containing 0.2M sodium chloride, 0.1M

MES, pH 6.0, 20% (w/v) PEG2000 MME, and equilibrated with 400 μ L of the same solution by the hanging drop method at 20°C. Crystals of HpThyX-FAD-dUMP with dimensions of 0.1 $\times$ 0.1 $\times$ 0.3 mm³ were thus obtained after 1 week. (The containing of FAD in enzyme will be stated in results part.) The crystals of Se-HpThyX-FAD-dUMP were achieved in a similar condition to that for HpThyX-FAD-dUMP.

Data collection and structure determination

Crystals of either HpThyX-FAD-dUMP or Se-HpThyX-FAD-dUMP were soaked in the mother liquor containing 20% glycerol and flash-frozen in liquid nitrogen. Diffraction data were collected at Shanghai Synchrotron Radiation Facility (SSRF) BL17U beamline and processed with HKL2000.²⁹ The structure was solved by the single wavelength anomalous dispersion method using phenix.³⁰ The relevant data collection statistics are summarized in Table I. Structure was refined by CNS³¹ and Phenix.³⁰ Model building was performed using the program COOT.³² The quality of the final model was validated by PROCHECK.³³ The statistics of structure refinement are listed in Table I. The coordinates of the HpThyX have been deposited in Protein Data Bank with accession number of 3N3Y. All the structure figures were produced by PyMOL.³⁴

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