Functional Analysis of the *Mycobacterium tuberculosis* FAD-Dependent Thymidylate Synthase, ThyX, Reveals New Amino Acid Residues Contributing to an Extended ThyX Motif^{∇†}

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A novel FAD-dependent thymidylate synthase, ThyX, is present in a variety of eubacteria and archaea, including the mycobacteria. A short motif found in all *thyX* genes, RHRX₇₋₈S, has been identified. The three-dimensional structure of the *Mycobacterium tuberculosis* ThyX enzyme has been solved. Building upon this information, we used directed mutagenesis to produce 67 mutants of the *M. tuberculosis* *thyX* gene. Each enzyme was assayed to determine its ability to complement the defect in thymidine biosynthesis in a Δ *thyA* strain of *Escherichia coli*. Enzymes from selected strains were then tested in vitro for their ability to catalyze the oxidation of NADPH and the release of a proton from position 5 of the pyrimidine ring of dUMP. The results defined an extended motif of amino acids essential to enzyme activity in *M. tuberculosis* (Y44X₂₄H69X₂₅R95H RX₇S105XRYX₉₀R199 [with the underlined histidine acting as the catalytic residue and the underlined serine as the nucleophile]) and provided insight into the ThyX reaction mechanism. ThyX is found in a variety of bacterial pathogens but is absent in humans, which depend upon an unrelated thymidylate synthase, ThyA. Therefore, ThyX is a potential target for development of antibacterial drugs.

The capacity to synthesize thymidine has been recognized as an essential function in all free-living organisms for many decades (13, 14, 19). This dependence has led to the development of a large number of inhibitors of thymidine synthesis. For most of this time, research focused on the enzyme that is common in eukaryotes and many eubacteria, the thymidylate synthase enzyme ThyA (EC 2.1.1.45), which reductively methylates dUMP in tandem with dihydrofolate reductase DHFR (EC 1.5.1.3) (3). More recently, a novel FAD-dependent thymidylate synthase, ThyX (EC 2.1.1.148), which transfers the methyl group without oxidation of the tetrahydrofolate carrier, has been identified in a variety of eubacteria and archaea (9, 22, 23). Different from ThyA proteins, ThyX proteins use a redox-activated FAD cofactor to form dTMP (Fig. 1A), but details of the reaction remain poorly understood. This is exemplified by the fact that four different reaction mechanisms have been proposed (Fig. 1B). Structural data exist for several ThyX proteins; however, since active site configurations in different structures vary we undertook an exhaustive mutagenesis study to identify amino acid residues indispensable for *Mycobacterium tuberculosis* ThyX activity.

In most organisms either the ThyA/DHFR couple or ThyX is utilized for thymidine biosynthesis. Like most eukaryotes, humans depend upon ThyA, so there has been special interest in the enzyme of ThyX-dependent pathogens such as *Helico-*

bacter pylori, *Campylobacter jejuni*, *Borrelia burgdorferi*, and *Treponema pallidum* (see reference 22 for a review). ThyX in these organisms is an attractive drug target (4, 6, 15, 23). Eubacterial species in the corynebacterium group are unusual because they have retained the genes for both the ThyA/DHFR and the ThyX pathways (22, 23). Within this group the sequences of the *thyA* genes are very highly conserved, and the *thyX* genes show almost complete identity (30). According to the high-density transposon mutagenesis study of *M. tuberculosis* H37Rv by Sasseti et al., *thyX* is an essential gene in *M. tuberculosis* whereas *thyA* is not (28).

The structures of the ThyX protein from *Thermotoga maritima* (16), *M. tuberculosis* (27), and *Paramecium bursaria* chlorella virus-1 (10) have been solved recently, and the basic structures are very similar. All three enzymes are homotetramers, and they are structurally and evolutionarily unrelated to any of the ThyA enzymes that have been studied (22, 23). The genes that encode the putative ThyX enzyme have been identified in organisms from several hundred species, representing more than 70 genera, and there are some highly conserved amino acids that have been termed the ThyX motif (RHRX₇₋₈S) (17, 18). Moreover, a limited study of essential residues in the *M. tuberculosis* ThyX enzyme confirmed that they were conserved in the orthologous *H. pylori* enzyme (17, 27).

Our goal in this study was to use directed mutagenesis of the *M. tuberculosis* *thyX* gene to identify a much wider range of amino acid residues essential to ThyX enzymatic function in cells and in two distinct in vitro assays. Essentially, we wanted to supplement the crystallographic studies with in-depth knowledge about the importance of individual amino acid residues. The various crystal structures available for ThyX proteins demonstrate considerable structural plasticity at the ac-

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FIG. 1. ThyX reactions and mechanisms. (A) Reactions catalyzed by ThyX enzymes. (B) Four currently proposed reaction mechanisms for ThyX, as outlined in reference 4.

tive configuration and also provide a static image of the active site during catalysis. However, *in vivo* catalysis is a dynamic process wherein configurational changes rearrange the active site, allowing different amino acid residues to access the substrates. We wanted to focus on identification of amino acid residues that are crucial to catalysis, which may or may not have been identified in the crystal structure. The strong conservation of the structure and the sequence of the *thyX* genes between *M. tuberculosis* and human pathogens that depend only upon ThyX suggest that the key residues identified in this work can be usefully extrapolated to the ThyX enzymes in those pathogenic bacteria as well.

MATERIALS AND METHODS

Expression vector cloning. *thyX* (Rv2754c) from *M. tuberculosis* H37Rv was cloned into a pUC18 vector for complementation analysis and into a pET24d vector with a six-His tag for high-level protein expression and purification as described previously (27). The cloned vectors are designated pUC18-ThyX and pET24d-ThyX, respectively.

Mutagenesis. To perform mutagenesis, primers that flank each of the target sites were designed (see Table S1 in the supplemental material). The primers were designed to anneal to the coding strand of DNA, and as such only one

primer was necessary for each point mutation. Mutations were generated using a QuickChange multisite-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. Potential mutant plasmids were sequenced completely on both strands to verify the point mutations. All mutations were made in *thyX* genes cloned into both the pUC18-ThyX and pET24d-ThyX vectors.

Complementation. An *Escherichia coli* strain lacking endogenous ThyX activity, χ 2913 (Δ thyA572 *recA56*), was graciously provided by R. Thompson, University of Glasgow, Scotland, United Kingdom (21). This *thyA*-negative *E. coli* strain is incapable of growth on M9 minimal medium plates without supplementation with thymidine. The χ 2913 strain was transformed with each pUC18-ThyX vector, containing either wild-type or mutated *thyX*, by use of the CaCl₂ method (5). Transformants were selected on LB agar plates with 100 μ g/ml of ampicillin and grown at 37°C. Colonies from each of the *thyX* mutants were streaked onto LB agar plates with ampicillin and grown overnight at 37°C. Each of the different ThyX mutated strains was again streaked onto two different M9 minimal medium plates, one without thymidine and the other with 50 μ g/ml of thymidine. Colonies were counted after 2 days at 37°C.

Protein expression and purification. *E. coli* BL21(DE3)/pLysS strains containing the six-His-tagged plasmids carrying either the wild-type or mutant *M. tuberculosis* *thyX* genes were grown overnight at 37°C in 50 ml of LB medium containing 30 μ g/ml of kanamycin and 35 μ g/ml of chloramphenicol. Five milliliters of this overnight culture was added to 500 ml of LB containing the same antibiotics and grown to an absorbance of 0.6 at 600 nm. Cells were induced with 1 mM IPTG (isopropyl- β -D-thiogalactopyranoside) for 3 h at 37°C. Cells were pelleted and lysed with lysozyme (1 mg/ml) and 1 mg/ml Pefabloc SC (1 mg/ml) (both from Sigma) in 1 $\times$ lysis equilibration wash buffer (3 ml solution/100 ml cells) from a Protino Ni-TED protein purification kit (Macherey-Nagel). Following sonication, lysates were spun at 18,000 $\times$ g for 1 h at 4°C. Supernatants were extracted and loaded into pre-equilibrated columns, and proteins were eluted from the Ni-TED columns provided in the Protino kits according to the manufacturer's instructions. To remove the imidazole from the eluates, eluted protein fractions were dialyzed overnight at 4°C using D-tube dialyzers (Midi molecular weight cutoff 3.5 kDa; EMD Biosciences) in a volume of 50 mM Tris-HCl, 10% glycerol, and 200 mM NaCl at pH 7.5, with a 1,000-fold excess of the sample.

Determination of higher-order protein complexes. To investigate whether mutant proteins were able to form higher-order complexes similarly to wild-type ThyX, 2- to 3- μ g aliquots of purified His-tagged ThyX proteins were analyzed on 4 to 20% gradient gels in a buffer containing 25 mM Tris-HCl and 250 mM glycine at pH 8.0 without any detergents or reducing agents added. The predicted pI of the *M. tuberculosis* ThyX protein is 6.51. We used a high-molecular-weight calibration kit for native electrophoresis purchased from Amersham as markers on the native gels. Each isolate was also analyzed on reducing 8% sodium dodecyl sulfate (SDS)-polyacrylamide gels to determine the size of the monomer. Precast polyacrylamide gels were purchased from NuSep.

NADPH oxidation assays. To measure the capacity of the mutant ThyX enzymes to oxidize NADPH, reactions were carried out in a total volume of 200 μ l with 14 to 15 μ g eluted ThyX protein added to a 50 mM HEPES, pH 7.5, solution containing 1 mM MgCl₂, 50 μ M dUMP, and 12.5 μ M FAD. Reactants were incubated for 5 min at room temperature before the oxidation reaction was started by addition of 0.2 mM β -NADPH. Oxidation of NADPH was measured by a decrease in absorbance at 340 nm in a Labsystems Multiscan 96-well plate reader. The amount of NADP⁺ produced by each ThyX enzyme was determined after the reaction proceeded for 3 min by use of an extinction coefficient (ϵ ₃₄₀) of 6,220 M⁻¹ cm⁻¹ (24).

Tritium release assays. To measure "deprotonation" of [5-³H]dUMP *in vitro*, tritium release assays were performed as described previously (17) by using 5 to 10 μ g ThyX protein, incubating at 37°C for 20 min, and then stopping the reactions with activated charcoal. Reactions were performed in a total volume of 50 μ l, and the mixtures contained 0.2 mM 5,10-methylene-5,6,7,8-tetrahydrofolate, 0.4 mM FAD, 0.1 mM β -NADPH, 0.1 mM β -NADH, 20 μ M nonradioactive dUMP, 0.6 μ Ci (0.92 μ M) [5-³H]dUMP (specific activity, 13.6 Ci/mmol; Moravsek), 10 μ M MgCl₂, and 50 mM HEPES, pH 7.5.

Reagents. All chemical reagents were purchased from Sigma except for the methylenetetrahydrofolate, which was generously provided to us by R. Moser (Merck Eprova AG).

RESULTS

To provide mechanistic insight into the NADPH oxidation and deprotonation activities of ThyX proteins, we examined in depth the previously defined ThyX motif RHRX₇₋₈S (17, 18).

We extended this analysis by testing residues within and surrounding the previously defined motif to identify amino acids required for full enzyme function and for oxidation and deprotonation activity in vitro. Since the structure of *M. tuberculosis* ThyX has been solved previously (27), we focused our attention on highly conserved amino acids and those shown to interact in crystals with the substrates and cofactors required for this complex reaction (Table 1). Figure 2 shows the alignment of this extended ThyX motif from several diverse eubacterial and archaeal species and highlights specific residues that are highly conserved.

Complementation. To test the function of certain amino acids within the extended ThyX motif, we performed site-directed mutagenesis on 51 amino acid residues in the coding region of *M. tuberculosis* thyX, creating 67 mutants, each containing one amino acid substitution (Table 1). We chose to test amino acid residues based on two criteria. These residues were either conserved among diverse eubacterial and archaeal species or implicated in forming hydrogen bonds with FAD or dUMP as shown from the crystal structure of *M. tuberculosis* ThyX (Table 1). It is surprising that not all of the FAD and dUMP binding residues in *M. tuberculosis* ThyX are conserved, which supports the need for further functional studies.

The enzymatic function of each mutant enzyme was tested by cloning into a pUC18 plasmid and transformation into *E. coli* χ 2913, a strain that does not contain a gene for ThyX and lacks endogenous ThyA activity (21). Following transformation, the number of transformants observed on minimal medium plates without thymidine supplementation was counted. Mutants were placed in one of three categories comparing the level of complementation of growth in the *E. coli* system with that observed with the wild-type plasmid. We defined full complementation as at least 80% of the wild-type number of colonies, low complementation as less than 5% of the number of wild-type ThyX colonies on minimal medium plates, and no complementation as those mutants that gave no colonies at all on minimal medium. Among the 67 mutants tested, 25 mutants complemented as efficiently as the wild-type *M. tuberculosis* ThyX, 35 failed to complement, and seven complemented at less than 5% the level of wild-type ThyX. No mutant enzymes that we tested had between 5 and 80% of the wild-type number of colonies. Table 1 shows the results of these studies.

Several amino acid residues of *M. tuberculosis* ThyX not included in the previously defined ThyX motif are of special interest. For example, Y44 makes one hydrogen bond to FAD via a water molecule. Two changes were made at this residue, one to phenylalanine to conserve the shape, hydrophobicity, and size of the residue and the other to alanine. Neither the Y44A nor the Y44F mutant complemented growth. The H69 residue has been suggested to function as a key catalytic residue and is highly conserved in all ThyX proteins, but its configuration varies in different structures. The H69 residue had previously been changed to glutamate to reverse the charge of the residue and failed to complement growth of the χ 2913 *E. coli* strain, which supports the catalytic role for this histidine residue (27). The residues H91 and E92 form one and two hydrogen bonds to dUMP, respectively. Proteins with either of the alanine mutations complemented at a very low level.

Structural data have suggested that some amino acid residues that interact with several substrates or cofactors during

ThyX catalysis are likely to undergo conformational changes during catalysis. The R95 residue interacts in crystals of *M. tuberculosis* ThyX with FAD via six hydrogen bonds and dUMP by three hydrogen bonds, and it is the first residue in the previously described ThyX motif. Since this arginine occupies a key area in the active site of the enzyme, three separate mutational changes were made to this residue. R95 was changed to a lysine to conserve both the charge and most of the shape of the residue, to an aspartic acid to reverse the charge, and finally to an alanine as a noncharged residue. As previously shown, the three mutant enzymes containing R95K, R95D, or R95A all failed to complement growth (27). Two other residues, Q103 and R199, interact in crystals with dUMP and FAD (see Fig. S1 in the supplemental material). The Q103 residue is the least conserved among the three residues that bind both dUMP and FAD. It forms one hydrogen bond to dUMP and two hydrogen bonds to FAD. The R199 residue is highly conserved and forms one hydrogen bond to FAD and three hydrogen bonds to dUMP. Proteins containing either alanine mutation did not complement.

A pair of histidines, H96 and H98, both form one hydrogen bond each to FAD. The H96 residue is highly conserved, but H98 is not conserved within the ThyX motif. Complementation still occurred when either histidine was changed to alanine; however, when both residues were converted to alanines complementation was abolished. This observation is analogous to a similar observation by Leduc et al. (17) indicating that at least one serine residue in the vicinity of the ThyX motif is required for *H. pylori* ThyX catalysis. The double mutant data shown above support the essentiality of at least one histidine residue in the ThyX motif, as demonstrated in previous work (11, 17). The R97 residue forms two hydrogen bonds to FAD and is highly conserved among ThyX proteins, and the R97A mutant protein did not complement growth. The S105 residue is a universally conserved nucleophile and forms two hydrogen bonds to dUMP. ThyA enzymes also transfer a methylene group to dUMP, and a cysteine functions as the nucleophile, so to test whether cysteine might function similarly in ThyX, S105 was changed to cysteine. The S105 residue had also previously been changed to glutamate to engineer a mutant with a less conserved residue (27). Both the S105C and S105E mutant proteins failed to complement. Both Q106 and R107 form two hydrogen bonds each to dUMP, but Q106 is not conserved whereas R107 is highly conserved among ThyX enzymes. The Q106A and the R107A mutant proteins also failed to complement.

Seven residues within the *M. tuberculosis* ThyX motif, F99, S100, Y101, S102, L104, Y108, and V109, have no known interactions with any substrate. Mutations had not previously been made in these residues, with the exception of the Y108F mutant (discussed briefly in reference 27). This Y108 residue is the only conserved amino acid among ThyX proteins from these seven residues, which is a phenylalanine in some species. ThyX mutant enzymes containing F99A, S100A, or L104A failed to complement. S100 was also changed to threonine to conserve the hydroxyl group; the S100T mutant was able to complement. Two mutants, the Y101A and V109E mutants, complemented at a level lower than wild-type ThyX but higher than the H91A and E92A mutant enzymes. The S102A mutant complemented growth. Two changes were made to Y108, one

TABLE 1. *M. tuberculosis* ThyX data

Wild-type or mutant enzyme	Interaction(s)	Complementation ^a	Oxidation ^b		% Tritium release ^c
			Rate (pmol mg ⁻¹ min ⁻¹)	%	
Wild-type ThyX	NA ^d	Yes	140.5 ± 8.1	100 ± 5.8	100
A39F	Unknown	No	— ^e	—	—
R41A	Unknown	Low	—	—	—
C43M	1 H-bond FAD	No	40.4 ± 23.0	28.8 ± 16.4	—
Y44A	1 H-bond FAD via water	No	28.5 ± 26.9	20.3 ± 19.1	2
Y44F	1 H-bond FAD via water	No	96.4 ± 4.5	68.6 ± 3.2	8
S46A	Unknown	No	24.5 ± 1.0	17.4 ± 0.7	3
L61F	Unknown	No	—	—	—
I65M	Unknown	Yes	—	—	—
H69E	Catalytic	No	24.3 ± 10.4	17.3 ± 7.4	—
S71A	1 H-bond FAD	Yes	95.0 ± 11.4	67.6 ± 8.1	—
S71T	1 H-bond FAD	Yes	—	—	—
L73F	Unknown	Yes	—	—	—
E74A	Unknown	No	34.0 ± 6.3	24.2 ± 4.5	—
E74N	Unknown	No	—	—	—
E74Q	Unknown	Yes	—	—	—
H75A	Unknown	No	—	—	—
F80Y	Unknown	Yes	—	—	—
R87A	3 H-bonds dUMP	No	14.3 ± 6.1	10.2 ± 4.3	4
S88A	Unknown	Yes	236.6 ± 21.8	168.4 ± 15.5	71
H91A	1 H dUMP	Low	35.2 ± 11.7	25.1 ± 8.3	1
E92A	2 H dUMP	Low	48.9 ± 21.3	34.8 ± 15.2	—
R95A	2 H-bonds dUMP and 6 H-bonds FAD	No	3.8 ± 3.5	2.7 ± 2.5	—
R95D	2 H-bonds dUMP and 6 H-bonds FAD	No	—	—	—
R95K	2 H-bonds dUMP and 6 H-bonds FAD	No	—	—	—
H96A	1 H-bond FAD	Yes	53.2 ± 31.3	37.9 ± 22.2	—
R97A	2 H-bonds FAD	No	11.5 ± 13.4	8.2 ± 9.5	5
H98E	1 H-bond FAD	Yes	105.4 ± 8.1	75.0 ± 5.8	94
H98K	1 H-bond FAD	Yes	82.8 ± 19.1	58.9 ± 13.6	58
F99A	Unknown	No	19.3 ± 3.2	13.7 ± 2.3	—
S100A	Unknown	No	78.0 ± 3.7	55.5 ± 2.6	89
S100T	Unknown	Yes	—	—	—
Y101A	Unknown	Low	50.2 ± 20.7	35.7 ± 14.7	13
S102A	Unknown	Yes	178.0 ± 24.8	126.7 ± 17.7	—
Q103A	1 H-bond dUMP and 2 H-bonds FAD	No	12.1 ± 3.3	8.6 ± 2.3	2
L104A	Unknown	No	187.8 ± 8.4	133.7 ± 6.0	165
S105C	2 H-bonds nucleophile	No	16.8 ± 17.2	12.0 ± 12.2	4
S105E	2 H-bonds nucleophile	No	1.0 ± 15.4	0.7 ± 11.0	—
Q106A	2 H-bonds dUMP	No	13.5 ± 6.6	9.6 ± 4.7	1
R107A	2 H-bonds dUMP	No	24.9 ± 26.9	17.7 ± 19.1	—
R107V	2 H-bonds dUMP	No	—	—	—
Y108F	Unknown	Yes	353.9 ± 12.8	251.9 ± 9.1	—
Y108S	Unknown	No	56.7 ± 6.6	40.4 ± 4.7	9
V109E	Unknown	Low	243.4 ± 9.6	173.2 ± 6.8	7
K165A	Unknown	No	2.0 ± 6.7	1.4 ± 4.8	4
R168A	Unknown	No	24.5 ± 2.2	17.4 ± 1.6	—
A171F	Unknown	No	—	—	—
R172A	2 H-bonds dUMP	No	40.1 ± 13.3	28.5 ± 9.5	2
L175M	Unknown	Yes	—	—	—
P176A	Unknown	No	—	—	—
T181A	Unknown	Low	230.4 ± 4.5	164.0 ± 3.2	22
T181F	Unknown	No	—	—	—
T181L	Unknown	No	—	—	—
T181S	Unknown	Yes	—	—	—
I183F	Unknown	Yes	—	—	—
N188A	1 H-bond FAD	Yes	209.5 ± 8.8	149.1 ± 6.3	—
R190A	3 H-bonds FAD	Yes	75.2 ± 6.3	53.5 ± 4.5	18
H194K	2 H-bonds FAD	Yes	44.9 ± 13.1	32.0 ± 9.3	35
F195V	Unknown	Low	144.9 ± 12.9	103.1 ± 9.2	1

Continued on following page

TABLE 1—Continued

Wild-type or mutant enzyme	Interaction(s)	Complementation ^a	Oxidation ^b		% Tritium release ^c
			Rate (pmol mg ⁻¹ min ⁻¹)	%	
F195Y	Unknown	Yes	—	—	—
M198V	Unknown	Yes	484.3 ± 9.9	344.7 ± 7.0	49
R199A	1 H-bond FAD and 3 H-bonds dUMP	No	28.9 ± 4.3	20.6 ± 3.1	1
H203E	Unknown	Yes	—	—	—
A204F	Unknown	No	—	—	—
D205A	Unknown	Yes	—	—	—
I208F	Unknown	Yes	—	—	—
I208M	Unknown	Yes	—	—	—
A212F	Unknown	No	—	—	1
Ins-317C ^f	—	No	—	—	—
I65M/L175M	Unknown	Yes	—	—	—
H96A/H98E	2 H-bonds FAD	No	—	—	—

^a Yes, mutant enzymes that produced over 80% the number of colonies on M9 plates as wild-type ThyX; low, mutant enzymes producing less than 5%; no, mutant enzymes producing 0%.

^b Wild-type ThyX activity at 140.5 pmol NADP⁺ produced/μg enzyme/min is set to 100%. Values are means ± standard deviations.

^c Wild-type ThyX activity is set to 100%.

^d NA, not applicable for wild-type protein.

^e —, the assay was not performed.

^f Ins-317C, Insertion of cytosine at 317 bp.

to a serine to conserve the hydroxyl group, and this Y108S enzyme failed to complement. However, this position is a phenylalanine in some species, and a Y108F mutant was able to complement growth.

This detailed analysis allowed us to identify many amino acids that are essential to ThyX enzyme activity. As one might expect, highly conserved amino acids were likely to be essential to enzyme function, and those residues that interacted closely with FAD and BrdUMP (5-bromo-dUMP) in the *M. tuberculosis* ThyX crystal structure were also essential. These data extend the list of amino acids that define an enzymatically active ThyX protein.

TB	YX ₂₄	HX ₂₅	RHRX ₇	SORYX ₉₀	R
ML	YX ₂₄	HX ₂₅	RHRX ₇	SORYX ₉₅	R
Cd	YX ₂₄	HX ₂₅	RHRX ₇	SORFX ₉₂	R
PB	YX ₂₇	HX ₂₄	RHRX ₇	SORYX ₉₀	R
Td	YX ₂₁	HX ₂₄	RHRX ₇	SGRYX ₉₄	R
Hp	WX ₂₅	HX ₂₅	RHRX ₇	SRRYX ₈₆	R
Ct	YX ₂₈	HX ₂₅	RHRX ₇	SORYX ₉₁	R
Cj	WX ₂₅	HX ₂₅	RHRX ₇	STRYX ₈₂	R
Hm	YX ₃₇	HX ₂₅	RHRX ₇	SMRYX ₁₀₀	R
Rt	YX ₂₁	HX ₂₄	RHRX ₇	SARYX ₁₀₆	R

FIG. 2. Extended ThyX motif alignments. The alignment of the extended ThyX motif among ThyX proteins from several organisms, some of which are pathogenic, including representative species from both eubacteria and archaea, was made using psi-BLAST. Conserved amino acid residues are in bold text, and highly conserved amino acid residues are in bold text surrounded by boxes. TB, *M. tuberculosis* H37Rv (GenBank accession no. P66930); ML, *Mycobacterium leprae* TN (GenBank accession no. Q9CBW3); Cd, *Corynebacterium diphtheriae* (GenBank accession no. Q6NGP3); PB, *Paramecium bursaria* chlorella virus-1 (GenBank accession no. O41156); Td, *Treponema denticola* (GenBank accession no. Q73QY2); Hp, *Helicobacter pylori* (GenBank accession no. O26061); Ct, *Clostridium tetani* (GenBank accession no. Q890M6); Cj, *Campylobacter jejuni* (GenBank accession no. Q5HDX2); Hm, *Haloarcula marismortui* (GenBank accession no. Q5V325); Rt, *Rickettsia typhi* (GenBank accession no. Q68X70).

Formation of higher-order complexes. In the active enzymatic state, wild-type *M. tuberculosis* ThyX protein forms tetramers. A mutant protein that prevented tetramer formation would certainly fail to be enzymatically active because residues from three of the four monomers contribute to each active site (17, 27). To determine whether noncomplementing mutant proteins were able to form tetramers, genes encoding each mutant protein were separately cloned into the pET24d protein expression vector and transformed into the χ 2913 *E. coli* strain. Proteins were expressed with six-histidine tags and isolated on nickel columns, and their migration on both native and reducing polyacrylamide gels was analyzed. High levels of protein were isolated from the wild type and all 67 mutant proteins. Figure 3A shows a representative SDS-polyacrylamide gel, and Fig. 3B shows a representative native polyacrylamide gel with wild-type protein and five mutant ThyX proteins containing the most extreme amino acid changes. On the native polyacrylamide gel, the five ThyX mutant proteins migrate similarly to the wild-type protein, which forms tetramers under native conditions. The high-molecular-mass band on the native polyacrylamide gels was probed with an antibody to the six-histidine tag, and Western blotting confirmed that this band was ThyX (data not shown). All 35 mutant ThyX proteins that failed to complement in the *E. coli* assay showed similar patterns on native polyacrylamide gels. We conclude that failure of complementation did not result from poor expression of the protein or from failure to form higher-order complexes, as expected for a tetrameric enzyme.

Oxidation assays. In ThyX enzymes, the conversion of dUMP to dTMP involves two half reactions, oxidation and transfer of the methylene group from dUMP to form the dTMP final product (1). To test the ability of the ThyX mutants to execute these essential half reactions, two biochemical assays were performed. First, the oxidation of NADPH was assayed for a subset of ThyX mutants (Table 1). We measured the sustained rate of oxidation of NADPH over a 3-min period with saturating amounts of the cofactor FAD (12.5 μM) and

FIG. 3. Mutant ThyX tetramer formation. (A) Eight-percent SDS-polyacrylamide gel showing, from left to right, wild-type *M. tuberculosis* ThyX and mutant proteins R95A and H98E, respectively, migrating at the correct monomeric size of *M. tuberculosis* ThyX (27.6 kDa). Lane 1 is a molecular mass marker. (B) Native 4 to 20% gradient polyacrylamide gel showing, from left to right, wild-type *M. tuberculosis* ThyX along with representative H69E, S105E, R95A, Q103A, and R199A mutant proteins, respectively, displaying high-molecular-mass bands near the correct tetramer size of *M. tuberculosis* ThyX (110.4 kDa). Lane 7, molecular mass marker.

substrates dUMP (50 μ M) and NADPH (0.2 mM). Overall, 14 of 39 mutant ThyX proteins oxidized NADPH at a rate greater than 50% that of wild-type ThyX. In fact, a subset of these mutant ThyX proteins—9 of 39—was able to oxidize NADPH at greater than 100% of the rate of wild-type ThyX (140.5 pmol NADP⁺ produced/ μ g enzyme/min). Figure 4 depicts the oxidation rates of NADPH by mutants within the extended ThyX motif grouped by their complementation status. Among the noncomplementing mutants, the Y44F, S100A, Y108S, and L104A mutants oxidized at 69%, 56%, 40%, and 134% of the wild-type rate, respectively. Rates for the other mutants were below 40%. Among the mutants that complemented, only the H96A mutant had a low oxidation rate at 38% that of the wild type whereas all other mutants oxidized NADPH in the same range as the wild type. Notably, the Y108F mutant had an

oxidation activity two to three times higher than the wild-type level (252%). Among the low-complementing group, the rates of the H91A, E92A, and Y101A mutants were considerably less than the wild-type rate (between 25 and 36%); however, the V109E mutant oxidized NADPH at a rate greater than the wild-type rate (173%).

Tritium release assays. The second assay used for the characterization of mutants measured the release of ³H from the 5-carbon position of the dUMP pyrimidine ring, a prerequisite for the transfer of the methyl group from methylenetetrahydrofolate to the dUMP substrate (1, 2, 23). In general, we selected mutant proteins that failed to complement in the *E. coli* ThyA knockout strain. These mutant proteins were then assayed for their ability to catalyze the release of a proton from dUMP. This reaction requires a nucleophilic attack on the

FIG. 4. Bar graph of NADPH oxidation rates among ThyX mutants. NADPH oxidation rates of a select group of ThyX mutants were assayed, and the rates of oxidation compared to that of the wild type (wt) are shown on the graph. Wild-type *M. tuberculosis* ThyX oxidized at a rate of 140.5 pmol NADP⁺ produced/ μ g enzyme/min, which is set to 100% in the figure. ThyX mutants were grouped into complementation categories based on whether they complemented, complemented less than 5% of the wild-type level, or failed to complement the *E. coli* ThyA knockout strain. The S102A, Y108F, and V109E mutants oxidized at rates of 127%, 252%, and 173%, respectively.

6-carbon position of dUMP that liberates the hydrogen at the 5-carbon position, thus preparing dUMP for the addition of a methylene group from methylenetetrahydrofolate to form dTMP. The tritium release assay measures the formation of tritiated water resulting from ThyX catalysis. Tritium release assays were performed on 15 mutant ThyX proteins which had failed to complement the χ 2913 *E. coli* strain (Table 1). Thirteen of these 15 mutant enzymes were also defective in this basic enzymatic function. To assay the ability of a cysteine to function as the nucleophilic residue in *M. tuberculosis* ThyX, tritium release was measured for the S105C mutant to be 4% of the wild-type rate. In general, the mutants that did not complement the *E. coli* ThyA knockout strain had tritium release rates less than 15% of the wild-type rate. Two non-complementing mutants, the Y44F and Y108S mutants, and two low-complementing mutants, the Y101A and V109E mutants, were all able to oxidize NADPH at modest or, in the case of the V109E mutant, substantial rates. These enzymes were also measured to have low tritium release rates of 8%, 9%, 13%, and 7%, respectively, compared with the wild-type rate. Finally, two noncomplementing mutant proteins, the S100A and L104A mutants, were both able to oxidize quite well and to perform tritium release at substantial rates (89% and 165% of the wild-type rate, respectively). Since they are unable to complement the *E. coli* ThyA knockout strain, their defect could lie for instance in the transfer of C₁ units or other later steps.

DISCUSSION

Thymidine biosynthesis is essential in virtually all organisms, but two different pathways have evolved to accomplish this critical task. The two thymidylate synthesis enzymes ThyA and ThyX are structurally and evolutionarily unrelated (16, 22, 23), but clearly they perform the same function. This similarity is illustrated most dramatically by the fact that the *M. tuberculosis* and *H. pylori* ThyX enzymes can both complement ThyA-deficient *E. coli* (17, 27). ThyA is used commonly in eukaryotes and in many microorganisms, including most eubacteria. ThyX has been identified more recently, and it is common among the archaea and some eubacteria, particularly those that are anaerobic or occupy extreme environments. The corynebacteria, the group that includes the mycobacteria, are unusual in having both ThyA and ThyX in their genomes. The extreme conservation of the *thyX* and *thyA* sequences within this group suggests that both genes serve essential functions for these organisms, but the roles for two parallel pathways are not yet defined. This study of the key residues in the *M. tuberculosis* ThyX enzyme is the first step toward understanding the function of this enzyme in the growth and various physiological states of this pathogen in its active and latent phases.

The classic pathway uses the ThyA enzyme to methylate dUMP in the final step of thymidine biosynthesis (3). In the alternative pathway, ThyX performs the transfer without oxidation of tetrahydrofolate, utilizing both dUMP and methylenetetrahydrofolate as reactants and FAD and NADPH in the redox phase of the reaction (1, 10, 17, 22, 23). Whereas ThyA requires a companion enzyme, dihydrofolate reductase, to regenerate tetrahydrofolate that has been oxidized in the transfer reaction, ThyX requires dUMP, NADPH, FAD, and

methylenetetrahydrofolate (3). Currently, four reaction mechanisms of ThyX have been proposed, as shown in Fig. 1B (1, 10–12). Our data have identified mutants (e.g., the Y44F, Y108S, V109E, R190A, F195V, and R199A mutants) that are still able to oxidize NADPH but are unable to catalyze the deprotonation reaction. This behavior is consistent with NADPH oxidation taking place before deprotonation of dUMP, as shown in Fig. 1B. Therefore, these analyses allowed identification of several residues that normally participate in coupling the NADPH oxidation and deprotonation reactions of ThyX. Some of these amino acid residues have interactions with FAD and/or dUMP, as shown in the crystallographic image of the *M. tuberculosis* ThyX active site (see Fig. S1 in the supplemental material).

We focused this study on amino acids that had been identified in two ways. First, alignment of the many eubacterial and archaeal *thyX* genes showed a set of highly conserved residues; these had been used to define a ThyX signature motif, RHRX_{7–8}S. The structure of the *M. tuberculosis* ThyX protein was solved with both FAD and BrdUMP bound, and this structure detailed important interactions of the protein and these molecules in crystals. Our goal was to test the validity of the ThyX motif for the *M. tuberculosis* enzyme and the importance of the molecular interactions observed in crystals.

A number of interesting mutants provided more guidance on the details of the enzyme activity. For example, several substitutions of amino acid residues with similar side groups successfully complemented the *E. coli* χ 2913 strain (E74Q, S100T, Y108F, and T181S) whereas the less conservative substitutions failed to complement (E74A, S100A, Y108S, and T181L). Moreover, there were mutants that failed to complement the *E. coli* χ 2913 strain that did catalyze the NADPH oxidation reaction in vitro (the Y44F, Y108S, F195V, and R199A mutants). There were no examples of mutants that catalyzed the ³H release but not the oxidation. The simplest explanation for this asymmetry is that the oxidation step of the reaction precedes deprotonation, which provides further support to the reaction mechanisms wherein NADPH oxidation precedes deprotonation (Fig. 1B) (10).

When the structures of *T. maritima*, *M. tuberculosis*, and *P. bursaria* chlorella virus-1 ThyX were solved, FAD was identified as being firmly bound within these ThyX enzymes (10, 16, 27). Furthermore, dUMP has been cocrystallized with *T. maritima* and *M. tuberculosis* ThyX (20) and NADPH was observed in the active site of *M. tuberculosis* ThyX (27); however, the position of the methylenetetrahydrofolate within the enzyme is not known. Under low-pH conditions (pH 4.5 to 5.5), NADPH was observed to displace FAD from preformed crystals in *M. tuberculosis* ThyX (26). Nevertheless, the fact that two of the residues (R190 and H194) that interacted with NADPH in the crystals also interacted with tightly bound FAD in the crystals (26, 27) indicates that the location of NADPH binding might not be biologically relevant. Two mutant proteins (the S100A and L104A mutants) were able to catalyze both the oxidation and the ³H release in vitro but still failed to complement the *E. coli* χ 2913 strain. This failure to complement is probably due to a defect in a later stage of the reaction possibly involving the transfer of C₁ units or the release of dTMP. These data provide the first clues to residues essential for the final steps of meth-

ylene transfer to the dUMP and/or release of the tetrahydrofolate.

In ThyA enzymes, a cysteine residue acts as the nucleophile by covalently binding the position 6-carbon of dUMP and stabilizing the deoxyribose ring (3, 25, 29). In all ThyX enzymes sequenced to date, a serine residue appears to function as the nucleophile and forms an integral part of the ThyX motif (see Table S2 in the supplemental material). The S105C mutant was unable to function as the nucleophile in ThyX, and this further underscores the fundamental differences between the two thymidylate synthase enzymes.

Several positions in the X₇₋₈ portion of the previously defined ThyX motif are not highly conserved among the *thyX* genes so far identified. It is likely that these serve to position key neighboring residues for their enzymatic function.

The previously defined motif was defined principally by alignment of the *thyX* genes so far identified (see Table S2 in the supplemental material), and our mutagenesis study has added to our understanding of the role of particular amino acids in the enzyme activity. Despite our work, it is still unclear which residues contribute to binding of methylenetetrahydrofolate and NADPH. Nonetheless, the combination of structural and mutagenesis studies reported here has provided a clearer picture of FAD and dUMP binding residues. Our analyses permit us to propose an extended range of the ThyX motif, shown here with the underlined histidine acting as the catalytic residue and the underlined serine as the nucleophile: (Y/W)X₁₉₋₄₀HX₂₅₋₂₈RHRX₇₋₈SXR(Y/F)X₆₈₋₁₁₄R.

The strong evolutionary conservation of the *thyX* gene suggests that our study of the *M. tuberculosis* enzyme will be useful for analysis of this enzyme with a wide range of human pathogens, including those that depend entirely on ThyX for biosynthesis of thymidine. Since this reaction is clearly essential in those organisms, the identification of inhibitors is a first step in developing drugs effective against them. When ThyX inhibitors are available, this work will aid in understanding their inhibitory effects. Because mycobacteria have both ThyA and ThyX pathways for thymidine synthesis, any effective drug might need to inhibit both enzymes. However, ThyA requires concomitant dihydrofolate reductase to regenerate tetrahydrofolate, so a combination of ThyX and DHFR inhibition might limit *M. tuberculosis* growth. Promising inhibitors of *M. tuberculosis* DHFR have been identified previously (7, 8). Our detailed determination of the function of key residues in the ThyX enzyme should facilitate further study of this potential drug target and will help to better understand the complex reactions of this interesting enzyme.

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