Published in final edited form as:

Arch Biochem Biophys. 2008 September 15; 477(2): 259–266. doi:10.1016/j.abb.2008.05.013.

Mechanistic analysis of *Mycobacterium tuberculosis* Rv1347c, a lysine *N*^ε-acyltransferase involved in mycobactin biosynthesis†

Brenda A. Frankel and John S. Blanchard*

Department of Biochemistry, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461, USA

Abstract

Mycobactin acylation plays a crucial role in the ability of *Mycobacterium tuberculosis* to acquire intracellular iron during infection. *M. tuberculosis* Rv1347c, the lysine *N*^ε-acyltransferase responsible for mycobactin acylation, represents a valid target for the development of novel anti-tubercular agents. Here we investigate the substrate specificity of Rv1347c, evaluate its kinetic mechanism and probe the contributions of active site residues to catalysis. Our results confirm that Rv1347c demonstrates a preference for longer acyl-chains and suggest that mycobactin acylation occurs subsequent to mycobactin core assembly. Steady-state bisubstrate kinetics and dead-end inhibitor studies support a random sequential kinetic mechanism. Analysis of the pH dependence of k_{cat}/K_m revealed the presence of two groups that must be deprotonated for efficient catalysis. Mutagenesis of His¹³⁰ and Asp¹⁶⁸ indicated that both residues are critical for acyltransferase activity and suggests that His¹³⁰ is responsible for general base activation of the ε-amino group of lysine.

Keywords

Rv1347c; lysine *N*^ε-acyltransferase; mycobactin; siderophore; GNAT; *Tuberculosis*

The rapidly emerging resistance of *Mycobacterium tuberculosis* to many frontline anti-microbials highlights the importance of identifying new targets for the development of effective anti-tubercular agents. In this regard mycobactin siderophores have recently garnered significant attention due to their role in the vital processes of iron acquisition and transport during infection by *M. tuberculosis* [1]. In the absence of siderophores, specifically mycobactins, *M. tuberculosis* growth is severely impaired in iron-depleted environments that mimic those encountered in the macrophages of the human lung during infection [2]. The biosynthesis of mycobactins is therefore directly linked to the virulence of *M. tuberculosis* and represents a valid target for the design of antimycobacterial compounds.

The predominant mycobactin in *M. tuberculosis*, mycobactin T (Figure 1), is a membrane-associated lipophilic siderophore synthesized in response to the limited availability of free iron in the host environment. This low molecular weight peptidic metabolite has several notable features [1,3]. A hydroxyphenyloxazoline moiety, derived from salicylate and serine, forms an *N*-cap on the mycobactin molecule and is linked by an amide bond to a hydroxylysine residue

†This work was supported by NIH grant AI60899 to JSB

*Corresponding author. Telephone: 718-430-3096 Fax: 718-430-8565, E-mail address: blanchar@aeom.yu.edu (J. Blanchard).

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

that is acylated on its ϵ -amino group. The acyl chain of lipophilic mycobactin T may contain 17 to 20 carbons; however, soluble carboxymycobactins have been isolated from *M. tuberculosis* with acyl chains that are 2 to 9 carbons in length and terminate either in a carboxylic acid or a methyl ester [1,3]. The remainder of the mycobactin molecule contains a 3-hydroxybutyric acid polyketide fragment and a second N^ϵ -hydroxylysine residue that is cyclized to form a seven-membered hydroxamate ring. Key to the function of mycobactin as a siderophore are six iron chelating ligands (bold in Fig 1) and the unusual *cis*-unsaturated acyl chain on the ϵ -amino group of the central hydroxylysine residue. The length and unsaturation of the acyl chain has been shown to modulate the membrane affinity of lipophilic mycobactins as well as their ability to directly acquire intracellular iron through lipid trafficking [4,5].

Although a number of genetic, structural, mechanistic and inhibitor design studies have focused on dissecting the hybrid NRPS/PKS biosynthetic machinery of the *M. tuberculosis* mycobactin core [1,2,6–14], relatively little emphasis has been placed on understanding the assembly, modification and attachment of the mycobactin acyl chain despite its undisputed importance in iron acquisition and transport. The isolation of didehydroxymycobactins (DDMs), and the preference of the lysine hydroxylase MbtG to hydroxylate acyl-lysine, suggest that lysine acylation may occur prior to hydroxylation [15,16]; however, there is a conspicuous lack of information regarding the sequence of acyl-chain dehydrogenation and transfer to the lysine ϵ -amino group relative to mycobactin core assembly. The organization of the iron-regulated *M. tuberculosis* gene cluster, *mbt-2*, encoding enzymes responsible for the assembly and attachment of the lipid moiety of mycobactin, suggests a sequential process for lipid assembly involving acyl chain activation, transfer to an acyl carrier protein, dehydrogenation, and attachment to the lysine ϵ -amino group [16].

Included within this newly identified *mbt-2* genetic locus is *rv1347c*, a gene previously identified as being essential for growth in *M. tuberculosis* [17] and originally annotated as an aminoglycoside acetyltransferase. Despite its original annotation, Rv1347c does not demonstrate aminoglycoside acetyltransferase activity [18] and rather displays sequence homology with bacterial homologues of IucB, a CoA-dependent N^ϵ -hydroxylysine *N*-acetyltransferase involved in the biosynthesis of the siderophore aerobactin [19]. Accordingly, Rv1347c has recently been shown to transfer acyl chains of varying length either from an acyl carrier protein (ACP) or from acyl-Coenzyme A (acyl-CoA) onto the ϵ -amino group of lysine as depicted in Scheme 1 [16]. The initial characterization of Rv1347c activity using a radio-TLC assay suggests a substrate preference for longer acyl chains over shorter ones and ACP-bound substrates over CoA-esters. Rv1347c was also found to demonstrate exclusive specificity for the ϵ -amino group of lysine while the α -amino position remained unmodified. Additionally, the δ -position of ornithine was reported to function as an alternate acceptor substrate for acyltransfer [16].

Structurally, Rv1347c belongs to the GCN5-related *N*-acetyltransferase (GNAT) family of acyltransferases [20]. Rv1347c possesses an acyl-CoA binding site, which is common to all GNATs, as well as an adjacent, and unique, hydrophobic channel that can accommodate an acyl chain of at least eight carbons. Two invariant residues, His¹³⁰ and Asp¹⁶⁸, are located in the putative active site of the enzyme and both are well positioned to play the role of a general base in deprotonating the amino acceptor group to activate it for nucleophilic attack on the thioacyl carbon of an acyl-CoA, a mechanism common to GNATs [21,22]. Modeling of N^ϵ -hydroxylysine into the Rv1347c structure revealed a potential hydrogen bonding interaction between His¹³⁰ and the N^ϵ -hydroxyl group, leading to the suggestion that His¹³⁰ functions to provide specific recognition of N^ϵ -hydroxylysine while Asp¹⁶⁸ is the general base that deprotonates the attacking nucleophilic nitrogen [20].

In this study we assess the substrate specificity of Rv1347c, evaluate its overall kinetic mechanism, and examine aspects of the chemical mechanism of acyltransfer. The results presented herein lay the framework for future studies aimed at elucidating the timing of lysine acylation with respect to other events in mycobactin biosynthesis and assigning specific roles to key active site residues.

MATERIALS AND METHODS

Materials

M. tuberculosis H37Rv genomic DNA was obtained from the TB Vaccine Testing and Research Materials Contract at Colorado State University. Primers were purchased from Invitrogen. Enzymes used in amplification and cloning were from New England Biolabs. *E. coli* strains TOP10 and BL21(DE3) pLysS were from Invitrogen and the pCRBlunt and pET15b plasmids were obtained from Invitrogen and Novagen, respectively. DNA sequencing was performed at the Albert Einstein College of Medicine DNA Sequencing Facility. Protein purification was performed on an AKTA Prime FPLC system (GE Healthcare). Lysine and acyl-coenzyme A substrates as well as buffers and salts were purchased from Sigma-Aldrich and used without further purification. Lys-OMe, Ornithine, Lys(Ac)-OH and sodium decanoate were also obtained from Sigma. Z-Lys-OMe was obtained from Novabiochem.

Cloning of *M. tuberculosis* Rv1347c

The *rv1347c* gene was amplified from *M. tuberculosis* genomic DNA using standard PCR conditions with the primers 5'-gctcgcccccatatgaccaaaccacatccgc-3' and 5'-cgatctggatccttacgcagccgtggcgga-3' containing the underlined NdeI and BamHI restriction sites, respectively. The PCR product was ligated into the cloning vector pCRBlunt (Invitrogen) and transformed into *E. coli* TOP10 cells. Colonies were screened for the presence of the *rv1347c* insert by digestion with NdeI and BamHI. The liberated gene was ligated into a similarly treated pET15b expression plasmid to generate a His₆-*rv1347c* construct. DNA sequencing confirmed the presence of the desired gene and the pET15b:*rv1347c* plasmid was transformed into *E. coli* BL21(DE3) pLysS cells for subsequent protein expression.

Overexpression and Purification of His₆-Rv1347c

An overnight culture of BL21(DE3) pLysS harboring the pET15b-*rv1347c* plasmid was used to inoculate two 1-L flasks of LB broth supplemented with ampicillin (100 µg/mL) and chloramphenicol (33 µg/mL). Cultures were grown with shaking at 37 °C to an OD₆₀₀ of ~0.5. The temperature was reduced to 18 °C and cultures were incubated for an additional 30–45 min prior to induction with IPTG (0.5 mM). After overnight growth, cells were harvested by centrifugation and resuspended in buffer A (20 mM Tris-HCl, 200 mM NaCl, 20 mM imidazole, pH 8.0). The cell suspension was incubated on ice for 30 min in the presence of EDTA-free Complete Protease Inhibitors (Roche), DNase I, and lysozyme. All subsequent steps were performed at 4 °C. Cells were lysed by sonication and the resultant lysate was clarified by centrifugation (34,500g for 40 min). The clarified lysate (50 mL) was loaded onto a 5-mL HisTrap column (GE Healthcare) equilibrated with buffer A. The column was washed with 100 mL of buffer A and Rv1347c was eluted with a 100 mL imidazole gradient (20 to 200 mM) at a flow rate of 4 mL/min. Fractions containing pure His₆-Rv1347c (as judged by SDS-PAGE) were pooled and concentrated to a 5-mL volume using a stirred cell concentrator (Amicon). The His₆ tag was cleaved with thrombin (approx. 1 unit/mg Rv1347c) during overnight dialysis against 2 L of buffer B (20 mM Tris-HCl, 100 mM NaCl, 2 mM CaCl₂, pH 8.0). Rv1347c was dialyzed for an additional 4 h against 2 L of buffer C (20 mM Tris-HCl, 100 mM NaCl, pH 8.0). After recovery from dialysis the protein solution was subjected to centrifugation (34,500g, 20 min) to remove traces of precipitate. Rv1347c concentration was measured using a calculated extinction coefficient ($\epsilon_{280} = 51910 \text{ M}^{-1}\text{cm}^{-1}$). Glycerol was

added to a final concentration of 10% and aliquots were frozen on dry ice prior to storage at -80°C .

Measurement of Enzyme Activity

Rv1347c acyltransferase activity was measured at 25°C in a 1-mL reaction volume containing Tris-HCl (100 mM, pH 8.5 or 9.0), DTNB (50 μM), lysine (untitrated or titrated to pH 8.5) and the indicated acyl-CoA substrate. The pH of the reaction mix was measured to be 8.5. Assays were initiated by the addition of Rv1347c (100 nM). Higher concentrations of Rv1347c (up to 1.45 μM) were sometimes necessary to measure activity with poor acceptor substrates. Activity was monitored continuously on a UVIKON XL spectrophotometer by following the increase in absorbance at 412 nm due to the reaction between the free sulfhydryl group of CoASH, generated by enzyme-catalyzed acyltransfer, and DTNB. Initial velocities were calculated from the measured absorbance change over a 0.5–2 min time frame using the extinction coefficient $13,600\text{ M}^{-1}\text{ cm}^{-1}$. In all experiments, enzymatic rates were corrected for the rate of uncatalyzed acyl-CoA hydrolysis recorded prior to the addition of enzyme. For this reason, the actual variability of kinetic parameters may be greater than the reported errors. It was estimated that acyl-CoA depletion due to uncatalyzed acyl-CoA hydrolysis did not exceed 10% of the starting acyl-CoA concentration.

Initial Velocity Experiments

The kinetic constants k_{cat} and K_{m} were obtained for each substrate studied by fitting initial velocity data to equation 1 using GraFit (Erithacus Software):

$$v = \frac{V_{\text{max}}S}{K_{\text{m}} + S} \quad (1)$$

where V_{max} is the maximal velocity, S is the concentration of the varied substrate and K_{m} is the Michaelis-Menten constant for S . The reported kinetic constants for acyl-CoA substrates were obtained using a fixed concentration of lysine (150 mM). Similarly, kinetic constants for lysine and lysine analogs were measured with a fixed concentration of decanoyl-CoA (300 μM). In all cases the reaction pH was measured to be 8.5.

To probe the kinetic mechanism of Rv1347c, intersecting line patterns obtained by simultaneously varying the concentration of decanoyl-CoA (20 to 300 μM) and lysine (20 to 150 mM) were fit to equation 2 describing a sequential reaction mechanism:

$$v = \frac{V_{\text{max}}AB}{K_{\text{ia}}K_{\text{b}} + K_{\text{b}}A + K_{\text{a}}B + AB} \quad (2)$$

where V_{max} is the maximal velocity, A and B are the concentrations of the substrates, K_{a} and K_{b} are the respective Michaelis-Menten constants, and K_{ia} is the inhibition constant for substrate A .

Inhibition Experiments

Sodium decanoate and ornithine were used as inhibitors in the Rv1347c-catalyzed acyltransfer reaction. Using the assay system described above, sodium decanoate (0 to 4 mM) and ornithine (0 to 300 mM) were varied simultaneously with decanoyl-CoA (20 to 300 μM) at a constant concentration of lysine (150 mM). In a separate set of experiments, sodium decanoate and ornithine were varied simultaneously with lysine (20 to 300 mM) at a constant concentration of decanoyl-CoA (100 μM).

The resulting inhibition patterns were fit using GraFit to equation 3 or equation 4 describing competitive and noncompetitive inhibition, respectively:

$$v = \frac{V_{\max} S}{S + K_m (1 + \frac{I}{K_i})} \quad (3)$$

$$v = \frac{V_{\max} S}{S (1 + \frac{I}{K_{ii}}) + K_m (1 + \frac{I}{K_{is}})} \quad (4)$$

where $V_{\max}$ is the maximal velocity, S is the concentration of the varied substrate, I is the concentration of inhibitor, K_m is the Michaelis-Menten constant for S , and K_{ii} and K_{is} are the intercept and slope inhibition constants for the inhibitor, respectively.

Dependence of Rv1347c Activity on pH

The pH dependence of Rv1347c activity was determined using decanoyl-CoA (10–400 μ M) as the variable substrate at a fixed concentration of lysine (150 mM). Activity was monitored from pH 7.1 to 9.5 using the buffers Tris-HCl (pH 7.1–8.5), sodium borate (pH 8.4–8.7) and sodium carbonate (pH 8.5–9.5), where reported pH values represent measurements of the assay mix starting pH. At lower pH, a higher concentration (200 nM) of Rv1347c was necessary for accurate measurement of reduced reaction rates. Initial velocity data were fit to equation 1 using GraFit to obtain k_{cat} and K_m values. The pH-rate profile was generated by plotting $\log k_{\text{cat}}/K_m$ as a function of pH and was fit to equations 5 and 6:

$$\log k_{\text{cat}}/K_m = \log \left(\frac{C}{1 + [\text{H}^+]/K_1} \right) \quad (5)$$

$$\log k_{\text{cat}}/K_m = \log \left(\frac{C}{1 + [\text{H}^+]/K_1 + [\text{H}^+]^2/K_1 K_2} \right) \quad (6)$$

where C is the pH-independent value of k_{cat}/K_m observed at high pH, $[\text{H}^+]$ is the concentration of hydrogen ions, and K_1 and K_2 are the ionization constants that describe the observed titrations.

Site-Directed Mutagenesis

The expression plasmid pET15b:*rv1347c* encoding wild-type His₆-Rv1347c served as a template for the introduction of single amino acid substitutions by PCR using the Quikchange method (Stratagene). All PCR reactions contained DMSO (5%). The complementary forward and reverse primer pairs used to create Rv1347c H130A and Rv1347c D168A are as follows, mutated codons are underlined: H130A fwd, 5'-cgatttggggctgcccgcggccatcgcg-3'; H130A rev, 5'-cgcgatggccgcccagccccaaatcg-3'; D168A fwd, 5'-cggatcatgttcgccccgatcaccgc-3'; D168A rev, 5'-gcggtgatcgggggcgaacatgatcg-3'. Mutational changes were confirmed by DNA sequencing.

RESULTS

Purification of Rv1347c

PCR amplification of *rv1347c* from *M. tuberculosis* H37Rv genomic DNA yielded a DNA fragment of the expected length (657 bp). The fragment was cloned into the pET15b expression vector and the identity of the *rv1347c* gene was confirmed by DNA sequencing. Expression of His₆-Rv1347c from the pET15b:*rv1347c* plasmid in *E. coli* BL21(DE3) pLysS cells resulted in the production, after purification, of 20 mg of soluble Rv1347c per liter of culture. As judged by SDS-PAGE, the purification procedure yielded an enzyme that was estimated to be >95% homogeneous and whose molecular weight was consistent with the mass of 24,080 Da expected for Rv1347c.

Initial Characterization of Rv1347c Activity

A previous study reported the use of Z-Lys-OMe as an effective mycobactin mimic in the acyltransferase reaction catalyzed by Rv1347c [16]. While this compound did prove to be a substrate in our assay system, low activity levels and limited substrate solubility made more extensive studies difficult. In contrast, when lysine was employed as a substrate for Rv1347c, we observed k_{cat} values that were 50-fold higher under the same assay conditions. With decanoyl-CoA and lysine as substrates, the K_m of Rv1347c for lysine was 190 ± 20 mM, and the k_{cat} for the reaction was $110 \pm 4 \text{ min}^{-1}$. The apparent K_m for decanoyl-CoA was measured to be $92 \pm 7 \text{ }\mu\text{M}$ when the lysine concentration was fixed at 150 mM.

Substrate Specificity of Rv1347c

The steady-state kinetic parameters for acyl-CoAs varying in chain-length from 6 to 16 carbons are summarized in Table 1. Consistent with previous reports [16], Rv1347c demonstrates a clear preference for longer chain lengths. Apparent K_m values of Rv1347c for fatty acyl-CoAs rapidly decrease as acyl chain length increases from 6 to 8 carbons and again from 8 to 10 carbons with the trend continuing through a chain length of 16 carbons. A 40-fold decrease in K_m was observed from hexanoyl to myristoyl-CoA, and although not as large, an increase in k_{cat} was also observed resulting in a 360-fold increase in k_{cat}/K_m as the acyl-chain length was varied from 6 to 14 carbons. When considering the data for palmitoyl-CoA (C_{16}), this trend is even more pronounced; however, caution must be used in interpreting this data as sensitivity limits prevented the measurement of activity with less than 0.5 μM palmitoyl-CoA, and apparent substrate inhibition due to micelle formation was observed at concentrations as low as 10 μM . Evaluating fatty acyl-CoAs longer than C_{16} was not feasible due to a combination of micelle formation and assay sensitivity limits. Shorter chain lengths were equally challenging as both K_m values and rates of uncatalyzed acyl-CoA hydrolysis increased. Accordingly, acetyl-CoA, up to a concentration of 1 mM, was not a substrate for Rv1347c.

Taking into consideration that the mycobactin T molecule possesses an *unsaturated* acyl-chain, two *trans*-unsaturated acyl-CoAs were assayed as substrates. Interestingly, neither 2-*trans*-octenoyl-CoA, up to a concentration of 0.97 mM, nor 2-*trans*-lauroyl-CoA, up to a concentration of 80 μM , showed any activity in the Rv1347c acyltransferase reaction.

Several lysine analogs, shown in Figure 2, were also tested for their ability to serve as substrates in the Rv1347c-catalyzed acyl transfer reaction. The introduction of benzyloxycarbonyl (Z) and methyl ester (OMe) capping groups to the lysine molecule, in the form of Lys-OMe and Z-Lys-OMe, produced decreases in both the observed k_{cat} and K_m parameters; however, k_{cat}/K_m did not change appreciably (Table 2). For Lys-OMe, a 9-fold reduction in k_{cat} was accompanied by a 2-fold drop in K_m . When the Z functionality was added in the form of the Z-Lys-OMe substrate, a further 6-fold reduction in k_{cat} , as well as a 12-fold reduction in K_m , was observed. While we anticipated that these additional substitutions might function to partially mimic the mycobactin scaffold, and enhance binding affinity, the observation that both k_{cat} and K_m decrease while k_{cat}/K_m remains relatively constant suggests that Lys-OMe and Z-Lys-OMe may exhibit nonproductive binding. Consistent with the known preference of Rv1347c to acylate the ϵ -amino group of the lysine side chain, Lys(Ac)-OH was not a substrate for Rv1347c when assayed at concentrations up to 300 mM. In addition, ornithine, although functional as an acceptor for acyl transfer, exhibited a 470-fold lower catalytic efficiency than lysine, highlighting the importance of the length of the lysine side chain in substrate recognition by Rv1347c.

Kinetic Mechanism

To determine the kinetic mechanism of Rv1347c, we performed a series of bisubstrate kinetic and inhibitor studies. Measuring the initial velocity of Rv1347c-catalyzed acyltransfer, while

simultaneously varying the concentrations of decanoyl-CoA and lysine substrates, resulted in the family of lines shown in Figure 3. The observed intersecting line pattern is diagnostic of a sequential kinetic mechanism, indicating the formation of a ternary Rv1347c-decanoylCoA-lysine complex along the reaction pathway.

A series of dead-end inhibition studies were performed to aid us in further distinguishing between random and ordered sequential mechanisms. We sought compounds that would serve as dead-end inhibitors versus lysine and decanoyl-CoA. Since Lys(Ac)-OH failed to serve as a substrate in the Rv1347c-catalyzed reaction, we predicted that it would be a competitive dead-end inhibitor versus lysine. Although Lys(Ac)-OH did prove to inhibit Rv1347c, inhibition was unexpectedly noncompetitive versus lysine as well as versus decanoyl-CoA (Table 3) and thus not useful in providing information regarding the order of substrate binding. In search of a competitive inhibitor for lysine, we tested the inhibitory properties of the weak substrate ornithine. We observed that ornithine inhibits Rv1347c-catalyzed acyltransfer at concentrations below that which are necessary to produce observable activity when employed as a substrate. As predicted, ornithine inhibition was competitive versus lysine; inhibition was also competitive versus decanoyl-CoA (Table 3).

With respect to decanoyl-CoA, we anticipated that while acetyl-CoA did not function as a substrate, it may act as a competitive inhibitor. However, when present in the assay at concentrations up to 1 mM, acetyl-CoA failed to inhibit Rv1347c and our ability to increase the AcCoA concentration was limited by high levels of background hydrolysis. Instead, we found that sodium decanoate was an effective mimic of decanoyl-CoA and inhibited Rv1347c activity. Sodium decanoate exhibited competitive inhibition versus decanoyl-CoA and noncompetitive inhibition versus lysine (Table 3). When considered in combination with the inhibition data for ornithine, these data suggest that Rv1347c utilizes a random sequential kinetic mechanism.

pH Dependence of Rv1347c Activity

To identify catalytically important ionizations and to gain insight into the mechanism of Rv1347c-catalyzed acyltransfer, we examined the pH dependence of catalysis. Due to the high K_m value measured for lysine, saturating concentrations of lysine were not achievable and we were therefore unable to examine the pH dependence of k_{cat} . The pH dependence of k_{cat}/K_m over the pH range from 7.1 to 9.5 is shown in Figure 4. An increase in the rate of uncatalyzed decanoyl-CoA hydrolysis at high pH limited the measurement of Rv1347c activity to this range. At pH 9.5, we estimate that no more than 10% of the decanoyl-CoA used in the assay was hydrolyzed nonenzymatically. The data were fit to both eq 5 (dashed line) and eq 6 (solid line) describing one or two ionizations, respectively. The data were most accurately described by a model involving the ionization of two distinct, yet overlapping, titratable groups which must both be deprotonated for optimal catalytic activity. Fitting the data to eq 6 gave pK_a values of 7.4 ± 0.1 and 8.9 ± 0.1 .

Site-Directed Mutagenesis

To further investigate the chemical mechanism of Rv1347c-catalyzed acyltransfer and to potentially identify the source of the catalytically important ionizations discussed above, two conserved active-site residues, His¹³⁰ and Asp¹⁶⁸ were mutated to alanine. Rv1347c H130A and Rv1347c D168A were cloned, expressed and purified as described for the wild-type enzyme. These mutants were found to be much less active than wild-type Rv1347c; however, the reaction of DTNB with cysteine residues on Rv1347c was too prominent at the elevated concentrations of mutant enzyme assayed to allow us to accurately quantify activity. We were only able to determine, based on raw initial velocity measurements, that Rv1347c H130A is at least 500-fold less active than wild-type Rv1347c, while the D168A variant is at least 300-

fold less active. In the hopes of creating a less impaired mutant enzyme which might allow us to make quantitative kinetic measurements, we replaced Asp¹⁶⁸ with glutamic acid. While this variant was more active than the other two mutants described, the concentrations of Rv1347c D168E needed to produce detectable rates were still too high to eliminate the significant contribution of the background DTNB reaction to measured velocities. We observed Rv1347c D168E to be at least 20-fold less active than wild-type enzyme. Although we were unable to quantify mutant activity, the fact that these mutations produced significantly impaired enzyme variants indicates that His¹³⁰ and Asp¹⁶⁸ are indeed important for catalysis.

DISCUSSION

The preference of Rv1347c for fatty acyl-CoAs with longer chain lengths (Table 1) suggests that Rv1347c is involved in synthesizing lipophilic mycobactin T and that an as yet unidentified acyltransferase with a preference for shorter chains may be responsible for lysine acylation in the case of soluble carboxymycobactins. The presence of a long hydrophobic channel in the structure of the enzyme, thought to be capable of binding an acyl chain of at least 8 carbons in length, supports this finding [20]. That acetyl-CoA was not a substrate for Rv1347c provides further support for the specific participation of Rv1347c in lipophilic mycobactin biosynthesis. However, our observation that acetyl-CoA was neither an Rv1347c substrate nor an inhibitor, suggests that in contrast to the acyl-chain moiety, the CoA portion of the molecule contributes little, if at all, to substrate binding despite the presence of a conserved acetyl-CoA binding site in the structure of Rv1347c [20]. This finding is consistent with the previous report that Rv1347c exhibits a 50-fold lower K_m for lauroyl-ACP compared to lauroyl-CoA [16]. Together with the presence of a neighboring ACP (Rv1344) in the *mbt-2* gene cluster, these observations suggest that the true *in vivo* acyl donor for Rv1347c-catalyzed acyltransfer is an acyl-ACP rather than an acyl-CoA.

The presence of an unusual *cis* double bond conjugated to the carbonyl group in the acyl chain of mycobactin T adds further intrigue since acyl-CoA dehydrogenases typically catalyze the formation of the *trans*-isomer. Either FadE14 (Rv1346), an acyl-CoA dehydrogenase homolog present in the *mbt-2* gene cluster, utilizes unique stereochemistry to directly catalyze the formation of the *cis*-isomer, or there is an as yet unidentified isomerase responsible for *cis* bond formation. Rv1347c has been reported to successfully process an ACP-tethered α,β -unsaturated lauryl chain, in which the unsaturation was introduced by FadE14, demonstrating that the FadE14 reaction product serves as a competent substrate for Rv1347c-catalyzed acyltransfer without intermediate processing [16]. In the current study, we report that neither 2-*trans*-octenoyl-CoA nor 2-*trans*-lauroyl-CoA are substrates for Rv1347c, illustrating the remarkable sensitivity of Rv1347c to α,β -unsaturation of the acyl chain and, since Rv1347c seems incapable of transferring *trans*-unsaturated chains, suggesting that FadE14 is likely to catalyze the formation of the *cis* isomer directly without the need for an isomerase.

Our studies of several lysine analogs as substrates for Rv1347c reveal millimolar K_m values for all lysine analogs tested (Table 2) leading us to believe that the ϵ -amino group of the lysine side chain may be acylated subsequent to incorporation into the mycobactin scaffold and/or hydroxylated by MbtG prior to acylation. The decreases in k_{cat} and K_m observed upon addition of Z and OMe groups suggest that substitutions at the α -amino and carboxyl groups of lysine affect productive Rv1347c substrate binding. Deacylated mycobactin, or a more appropriate peptidic mimic of the full mycobactin scaffold, may therefore function as a better substrate with a higher catalytic efficiency (k_{cat}/K_m). In an effort to test this theory we synthesized the synthetic peptide Ac-Thr-Lys-Ala-OH, which at available concentrations was not a substrate in the Rv1347c reaction. Future investigations involving the ability of other, more complex, synthetic scaffolds to serve as acyl-acceptor substrates may shed more light on the substrate specificity of Rv1347c and the relative order of lysine acylation and mycobactin core assembly.

Additionally, ornithine, whose side chain is shorter than lysine by only one carbon, was a very poor substitute for lysine, emphasizing the remarkable specificity of Rv1347c and suggesting that the length of the lysine side chain is crucial both for recognition and catalysis by Rv1347c.

We also assayed Lys(Ac)-OH as a substrate for Rv1347c. Although Lys(Ac)-OH is already acetylated at the ϵ -amino group, the existing modification does not preclude further acylation of this site since the ϵ -amino group of lysine is known to be doubly modified (acylated and hydroxylated) in mycobactin and doubly or triply modified (i.e. methylated) in histone tails. In this case however, Lys(Ac)-OH failed to serve as a substrate for Rv1347c. This finding suggests that N^ϵ -hydroxylysine is also not likely to be a substrate for Rv1347c despite structural modeling inferences that His¹³⁰ may function to provide specific recognition of the hydroxyl group of N^ϵ -hydroxylysine. Hydroxylation of lysine by MbtG is therefore likely to occur subsequent to acylation as suggested previously [15,16].

All GNATs characterized to date have been shown to utilize a sequential mechanism involving direct acetyl transfer from acyl-CoA to the amine acceptor substrate through the formation of a tetrahedral intermediate [21]. This mechanism requires that the acyl donor and amine acceptor substrates form a ternary complex with the enzyme before acyltransfer can occur. The intersecting initial velocity pattern shown in Figure 3 reveals that Rv1347c is no exception to this rule and catalyzes acyltransfer using a sequential mechanism. GNATs typically exhibit either a random kinetic mechanism or an ordered mechanism with the CoA substrate binding first [21]. To determine the order of substrate binding, we performed dead-end inhibition studies (Table 3). Inhibition by Lys(Ac)-OH was expected to be competitive versus lysine; instead, inhibition was noncompetitive versus both lysine and decanoyl-CoA. This result suggests that Lys(Ac)-OH may be capable of binding to more than one site within Rv1347c and highlights that a key structural determinant of substrate, and inhibitor, specificity may be absent in our assay system. Inhibition by ornithine was competitive versus both lysine and decanoyl-CoA, this is consistent with a random kinetic mechanism where there is an overlap between decanoyl-CoA and ornithine binding sites that prevents the formation of a ternary enzyme-decanoyl-CoA-ornithine complex [23]. Sodium decanoate inhibition was competitive versus decanoyl-CoA and noncompetitive versus lysine, also consistent with a random mechanism in which either substrate may bind to the enzyme first (Figure 5).

The pH dependence of Rv1347c catalysis provides some insight into acid/base involvement in the acyltransfer reaction. The pH dependence of k_{cat}/K_m revealed the presence two groups that must be deprotonated for optimal catalytic activity with $\text{p}K_a$ values of 7.4 ± 0.1 and 8.9 ± 0.1 (Figure 4). The $\text{p}K_a$ value of 8.9 is consistent with the expected $\text{p}K_a$ of the α -amino group of lysine. The α -amine of lysine is protonated at neutral pH but in the mycobactin scaffold, this group is an uncharged amide. A positive charge at this position could lead to misorientation and nonproductive binding of the lysine substrate within the Rv1347c active site and thus lead to the observation that it must be deprotonated for efficient catalysis. The second group with $\text{p}K_a$ 7.4 likely acts as a general base to deprotonate the ϵ -amino group of the lysine side chain to activate it for nucleophilic attack on the sulfur atom of the acyl-CoA substrate (Figure 5). In Rv1347c it was previously suggested that both His¹³⁰ and Asp¹⁶⁸ are well-positioned to function in this capacity [20]. In addition to general base activation of the amine nucleophile, many GNATs possess an acidic tyrosine residue that functions to protonate the thiolate anion of CoA following collapse of the tetrahedral intermediate [21]. An analogous tyrosine residue is absent in the structure of Rv1347c and it has been suggested that Thr¹⁷⁶ may function similarly [20]. However, our pH-activity studies provide no evidence for the participation of an acid in catalysis and it is possible that protonation of CoA is either not necessary, given the high pH optimum of the reaction, or is solvent mediated.

To delve more deeply into the catalytic mechanism of Rv1347c and in the hopes of definitively identifying the catalytic base, we performed site-directed mutagenesis on Asp¹⁶⁸ and His¹³⁰. However, activity was so severely diminished upon mutation of Asp¹⁶⁸ to either alanine or glutamic acid and of His¹³⁰ to alanine that we were unable to definitively assign specific catalytic roles for either of these residues on the basis of these experiments. Regardless, it seems clear from the dramatic decrease in activity upon side chain substitution, that both Asp¹⁶⁸ and His¹³⁰ are critical for Rv1347c activity and that one of these residues is likely to function as a general base to activate the lysine nucleophile.

Based on the positioning of amino acid side chains relative to an *N*^ε-hydroxylysine residue modeled into the Rv1347c active site, it was concluded that Asp¹⁶⁸ is the general base that deprotonates the attacking nucleophilic nitrogen while His¹³⁰ provides specific recognition of *N*^ε-hydroxylysine [20]. Although this hypothesis is consistent with the catalytic mechanisms observed for the majority of GNATs which utilize an Asp or Glu general base [21], we favor an interpretation in which His¹³⁰ of Rv1347c functions as the catalytic base to activate the lysine nucleophile and is responsible for the observed p*K*_a of 7.4 (Figure 5).

Several lines of reasoning support this model. First, we have demonstrated that *N*^ε-hydroxylysine is not likely to be a substrate for Rv1347c, calling into question the validity of making mechanistic conclusions based on modeled structural interactions of this molecule within the active site of Rv1347c. Second, in the absence of *N*^ε-hydroxylysine we observe a greater than 500-fold decrease in activity upon mutation of His¹³⁰ to alanine, suggesting a central role for His¹³⁰ in catalysis that is independent of *N*^ε-hydroxylysine recognition. Third, the p*K*_a value of 7.4 observed in the pH dependence of *k*_{cat}/*K*_m is consistent with the expected p*K*_a of a histidine side chain. Finally, a structural comparison of the active sites of Rv1347c and the closely related GNAT aminoglycoside acetyltransferase AAC(6')Ib, shows that His¹³⁰ of Rv1347c superimposes with the proposed catalytic base Asp¹¹⁵ of AAC(6')Ib (Figure 6, Vetting et al., submitted). Furthermore, the participation of a histidine residue as a general base in an acyltransfer reaction is not without precedent. Serotonin acetyltransferase employs a pair of histidine residues that function redundantly through intervening water molecules [24] and chloramphenicol acetyltransferase takes advantage of a histidine base in a mechanistically similar reaction [25].

While we cannot conclusively rule out the possibility that Asp¹⁶⁸ functions as the catalytic base in Rv1347c-catalyzed acyltransfer, it is clear, based on alignment with AAC(6')Ib that His¹³⁰ is positioned closer to proposed site of chemistry while Asp¹⁶⁸ is further removed. In AAC(6')Ib, Asp¹⁵² plays a role in aminoglycoside coordination. A similar role for Asp¹⁶⁸ of Rv1347c in mycobactin coordination is possible, although it is perhaps more likely that Asp¹⁶⁸ enhances the basicity of His¹³⁰ or stabilizes the protonated imidazolium ion following proton abstraction.

The studies reported here confirm the preference of Rv1347c for longer-length acyl-chains, begin to narrow down the issues of reaction sequence and substrate identity in the assembly of mycobactin, and provide insight into the kinetic and catalytic mechanisms of acyltransfer. In the future, structural information regarding specific contacts made between Rv1347c and bound substrates and/or inhibitors will be invaluable for deciphering the catalytic mechanism of the enzyme and designing selective inhibitors for mycobactin biosynthesis.

ABBREVIATIONS

ACP, acyl carrier protein
CoA, coenzyme A
DDM, didehydroxymycobactin

DMSO, dimethyl sulfoxide
 DTNB, 5, 5'-dithiobis-(2-nitrobenzoic acid)
 EDTA, ethylenediaminetetraacetic acid
 GNAT, GCN5-related *N*-acetyltransferase
 IPTG, isopropyl thio- β -D-galactosidase
 LB, Luria broth
 Lys(Ac)-OH, *N*^ε-acetyl-lysine
 NRPS, nonribosomal peptide synthetase
 PCR, polymerase chain reaction
 PKS, polyketide synthetase
 SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis
 TLC, thin-layer chromatography
 Z-Lys-OMe, benzyloxycarbonyl lysine methyl ester

ACKNOWLEDGMENTS

We thank Dr. James Errey for cloning Rv1347c, Dr. Matthew Vetting for preparation of Figure 6, and Dr. Patrick Frantom for insightful scientific discussions.

REFERENCES

1. De Voss JJ, Rutter K, Schroeder BG, Barry CE 3rd. *J. Bacteriol* 1999;181:4443–4451. [PubMed: 10419938]
2. De Voss JJ, Rutter K, Schroeder BG, Su H, Zhu Y, Barry CE 3rd. *Proc. Natl. Acad. Sci. U S A* 2000;97:1252–1257. [PubMed: 10655517]
3. Ratledge C. *Tuberculosis (Edinb)* 2004;84:110–130. [PubMed: 14670352]
4. Luo M, Fadeev EA, Groves JT. *Nat. Chem. Biol* 2005;1:149–153. [PubMed: 16408019]
5. Xu G, Martinez JS, Groves JT, Butler A. *J. Am. Chem. Soc* 2002;124:13408–13415. [PubMed: 12418892]
6. Crosa JH, Walsh CT. *Microbiol. Mol. Biol. Rev* 2002;66:223–249. [PubMed: 12040125]
7. Quadri LE, Sello J, Keating TA, Weinreb PH, Walsh CT. *Chem. Biol* 1998;5:631–645. [PubMed: 9831524]
8. Harrison AJ, Yu M, Gardenborg T, Middleditch M, Ramsay RJ, Baker EN, Lott JS. *J. Bacteriol* 2006;188:6081–6091. [PubMed: 16923875]
9. Zwahlen J, Kolappan S, Zhou R, Kisker C, Tonge PJ. *Biochemistry* 2007;46:954–964. [PubMed: 17240979]
10. Miethke M, Bissleret P, Beckering CL, Vignard D, Eustache J, Marahiel MA. *Febs J* 2006;273:409–419. [PubMed: 16403027]
11. Somu RV, Boshoff H, Qiao C, Bennett EM, Barry CE 3rd, Aldrich CC. *J. Med. Chem* 2006;49:31–34. [PubMed: 16392788]
12. Vannada J, Bennett EM, Wilson DJ, Boshoff HI, Barry CE 3rd, Aldrich CC. *Org. Lett* 2006;8:4707–4710. [PubMed: 17020283]
13. Duerfahrt T, Eppelmann K, Muller R, Marahiel MA. *Chem. Biol* 2004;11:261–271. [PubMed: 15123287]
14. Qiao C, Gupte A, Boshoff HI, Wilson DJ, Bennett EM, Somu RV, Barry CE 3rd, Aldrich CC. *J. Med. Chem* 2007;50:6080–6094. [PubMed: 17967002]
15. Moody DB, Young DC, Cheng TY, Rosat JP, Roura-Mir C, O'Connor PB, Zajonc DM, Walz A, Miller MJ, Levery SB, Wilson IA, Costello CE, Brenner MB. *Science* 2004;303:527–531. [PubMed: 14739458]
16. Krithika R, Marathe U, Saxena P, Ansari MZ, Mohanty D, Gokhale RS. *Proc. Natl. Acad. Sci. U S A* 2006;103:2069–2074. [PubMed: 16461464]
17. Sassetti CM, Boyd DH, Rubin EJ. *Mol. Microbiol* 2003;48:77–84. [PubMed: 12657046]

18. Draker KA, Boehr DD, Elowe NH, Noga TJ, Wright GD. *J. Antibiot. (Tokyo)* 2003;56:135–142. [PubMed: 12715873]
19. de Lorenzo V, Bindereif A, Paw BH, Neilands JB. *J. Bacteriol* 1986;165:570–578. [PubMed: 2935523]
20. Card GL, Peterson NA, Smith CA, Rupp B, Schick BM, Baker EN. *J. Biol. Chem* 2005;280:13978–13986. [PubMed: 15695811]
21. Vetting MW, LP SdC, Yu M, Hegde SS, Magnet S, Roderick SL, Blanchard JS. *Arch. Biochem. Biophys* 2005;433:212–226. [PubMed: 15581578]
22. Dyda F, Klein DC, Hickman AB. *Annu. Rev. Biophys. Biomol. Struct* 2000;29:81–103. [PubMed: 10940244]
23. Cook, PF.; Cleland, WW. *Enzyme Kinetics and Mechanism*. New York: Garland Science Publishing; 2007. ed.
24. Scheibner KA, De Angelis J, Burley SK, Cole PA. *J. Biol. Chem* 2002;277:18118–18126. [PubMed: 11884405]
25. Lewendon A, Shaw WV. *Biochem. J* 1993;290(Pt 1):15–19. [PubMed: 8439283]

Figure 1. Structure of *M. tuberculosis* mycobactin T. For lipophilic mycobactin T, R = CH₃ and n = 14–17. For soluble carboxymycobactin T, R = COOH or COOCH₃ and n = 0–6. The six iron-chelating ligands are shown in bold.

Figure 2.
Structures of lysine analogs used in this study.

Figure 3.

Bisubstrate kinetic analysis of Rv1347c acyltransfer. The concentrations of lysine and decanoyl-CoA were simultaneously varied in the spectrophotometric Rv1347c activity assay. The symbols are experimentally determined initial velocities, while the lines were obtained by global fitting of the data to eq 2 giving $V_{\max} = 33.1 \pm 0.1 \mu\text{M}/\text{min}$, $K_a = 430 \pm 2 \mu\text{M}$, $K_b = 520 \pm 1 \text{ mM}$ and $K_{ia} = 36.2 \pm 0.4 \mu\text{M}$. Shown is a double-reciprocal plot of initial velocity vs. decanoyl-CoA concentration at 4 fixed lysine concentrations: 20 ($\circ$), 40 ($\bullet$), 80 ($\square$) and 150 ($\blacksquare$) mM.

Figure 4.

pH dependence of Rv1347c activity. Shown is a plot of $\log k_{cat}/K_m$ versus pH. Activity was measured as a function of pH in Tris-HCl (●), sodium borate (■) or sodium carbonate (▲) buffers. The symbols are experimentally determined values while lines were obtained by fitting the data to eq 5 (■) or eq 6 (□). Fitting to eq. 6 indicates that two ionizable groups with pK_a 's of 7.4 ± 0.1 and 8.9 ± 0.1 must be deprotonated for optimal catalytic efficiency.

Figure 5.

Proposed kinetic and chemical mechanisms for Rv1347c-catalyzed acyltransfer. (A) Rv1347c catalyzes acyltransfer using a random sequential kinetic mechanism in which either acyl-CoA or lysine can bind first. (B) His¹³⁰ serves as the catalytic base to abstract a proton from the ϵ -amino group of lysine and activate it for nucleophilic attack on the thioester of acyl-CoA. Asp¹⁶⁸ stabilizes the positively charged imidazolium ion. The reaction proceeds through a zwitterionic tetrahedral intermediate which collapses to yield acylated lysine and CoA.

Figure 6.

Stereoview of Rv1347c active site residues superimposed on the active site of AAC(6')Ib. Rv1347c (PDB ID 1yk3) and AAC(6')Ib (PDB ID 2vqy) align with an RMSD of 1.27 Å over 132 common Cα atoms. The active site side chains of the aminoglycoside acetyltransferase AAC(6')Ib are shown in yellow with the aminoglycoside inhibitor paromomycin (PAR) and acetyl-CoA (AcCoA) substrate shown in green. The active site residues of Rv1347c are superimposed in gray. His¹³⁰ of Rv1347c overlays with Asp¹¹⁵ of AAC(6')Ib, which is poised to serve as a general base in the acetyltransfer reaction. Asp¹⁶⁸ of Rv1347c corresponds to Asp¹⁵² in AAC(6')Ib which is proposed to play a role in aminoglycoside substrate coordination.

Scheme 1.

Table 1

Acyl chain-length specificity for Rv1347c.

Acyl-CoA	k_{cat} (min^{-1}) ^a	K_{m} (μM) ^a	$k_{\text{cat}}/K_{\text{m}}$ ($\text{M}^{-1}\text{s}^{-1}$)
Hexanoyl	6.7 ± 0.6	1230 ± 260	90
Octanoyl	11.3 ± 0.4	440 ± 40	430
Decanoyl	78 ± 2	92 ± 7	1.4×10^4
Lauroyl	38 ± 2	52 ± 7	1.2×10^4
Myristoyl	52 ± 3	27 ± 4	3.3×10^4
Palmitoyl ^b	23.9 ± 0.4	0.27 ± 0.03	1.4×10^6

^a Apparent k_{cat} and K_{m} values measured in the presence of 150 mM lysine at pH 8.5. The actual variability in kinetic parameters may be greater than the reported errors due to correction for uncatalyzed acyl-CoA hydrolysis.

^b Activity not measurable with less than 0.5 μM Palmitoyl-CoA due to detection limits.

Table 2

Lysine substrates for Rv1347c.

Lysine analog	k_{cat} (min^{-1}) ^a	K_{m} (mM) ^a	$k_{\text{cat}}/K_{\text{m}}$ ($\text{M}^{-1}\text{s}^{-1}$)
Lysine	110 ± 4	190 ± 20	9.5
Lys-OMe	12.0 ± 0.3	95 ± 6	2.1
Z-Lys-OMe	1.96 ± 0.08	8 ± 1	4.1
Ornithine	0.70 ± 0.04	620 ± 90	0.02
Lys(Ac)-OH	n.a.	n.a.	n.a.

^a k_{cat} and K_{m} measured in the presence of 300 μM decanoyl-CoA at pH 8.5. The actual variability in kinetic parameters may be greater than the reported errors due to correction for uncatalyzed acyl-CoA hydrolysis.

Table 3

Summary of Rv1347c dead-end inhibition studies.

Inhibitor	Varied Substrate	Fixed Substrate	Inhibition Pattern	K_{is} (mM) ^a	K_{ii} (mM) ^a
Lys(Ac)-OH	lysine	dec-CoA	NC	40 ± 2	87 ± 5
Lys(Ac)-OH	dec-CoA	lysine	NC	33.3 ± 0.1	149 ± 1
Ornithine	lysine	dec-CoA	C	196 ± 7	n.a.
Ornithine	dec-CoA	lysine	C	200 ± 10	n.a.
NaDecanoate	lysine	dec-CoA	NC	1.7 ± 0.2	0.90 ± 0.04
NaDecanoate	dec-CoA	lysine	C	0.66 ± 0.06	n.a.

^a obtained by fitting initial velocity data to eq 3 or 4.