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Identification of amino acids involved in catalytic process of *M. tuberculosis* GlmU acetyltransferase

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Abstract *M. tuberculosis* GlmU is a bifunctional enzyme with acetyltransferase activity in C-terminus and uridyltransferase activity in N-terminus, and it is involved in the biosynthesis of glycosyl donor UDP-*N*-acetylglucosamine (UDP-GlcNAc). The crystal structure of *M. tuberculosis* GlmU clearly determines the active site and catalytic mechanism of GlmU uridyltransferase domain but not succeed in GlmU acetyltransferase domain. Sequence comparison analysis revealed highly conserved amino acid residues in the C-terminus between *M. tuberculosis* GlmU and GlmU enzymes from other bacteria. To find the essential amino acids related to *M. tuberculosis* GlmU acetyltransferase activity, we substituted 10 conserved amino acids in the acetyltransferase domain of *M. tuberculosis* GlmU by site-directed mutagenesis. All the mutant GlmU proteins were largely expressed in soluble and purified by affinity chromatography. Enzyme assays showed that K362A, H374A, Y398A and W460A mutants abolished more than 90 % activity of *M. tuberculosis* GlmU acetyltransferase and totally lost the affinity with two substrates, suggesting the potential substrate-binding functions. However, K403A, S416A, N456A and E458A mutants exhibited decreased GlmU acetyltransferase activity and lower kinetic parameters, probably responsible for substrate releasing by conformation shifting.

Keywords *Mycobacterium tuberculosis* · GlmU · Acetyltransferase · Site-directed mutagenesis · Kinetics

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Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (Mtb), is one of the main infectious diseases. The emergence of multi-drug resistant tuberculosis (MDR-TB) and extensively drug resistant tuberculosis (XDR-TB) [1] urgently demanded exploring new anti-TB drugs.

M. tuberculosis GlmU is a bifunctional enzyme catalyzing acetylation and uridylation reactions for the formation of UDP-*N*-acetylglucosamine (UDP-GlcNAc), the precursor of peptidoglycan and the sugar donor for disaccharide linker [2–4]. GlmU acetyltransferase activity acetylates glucosamine-1-phosphate (GlcN-1-P) to *N*-acetylglucosamine-1-phosphate (GlcNAc-1-P) using acetyl coenzyme A (Acetyl CoA), and its uridyltransferase activity transfers UDP to GlcNAc-1-P, generating UDP-GlcNAc. Previous studies on *M. smegmatis* and *M. tuberculosis* have confirmed that GlmU is a good drug target for mycobacteria [5, 6]. The kinetic properties of *M. tuberculosis* GlmU were determined using DTNB and malachite coupled colorimetric assays, respectively [7]. Since the acetylation reaction catalyzed by *M. tuberculosis* GlmU is not found in human, it is more significant to study *M. tuberculosis* GlmU acetyltransferase as a valuable drug target.

To date, although the crystal structure of *M. tuberculosis* GlmU uridyltransferase domain has been measured in different substrate-bound forms [8], the crystals of GlmU acetyltransferase complex with different substrates are not available

[9, 10]. Therefore, the amino acid residues located in the active site of GlmU acetyltransferase of *M. tuberculosis* remain uncertain. According to the crystal structure of *M. tuberculosis* GlmU in C-terminus, the LβH domain is composed of 231 residues (264–473) containing 10 repeats (10 turns) and an extended C-terminal tailing as a disordered region [9]. In our study, amino acids sequences of GlmU from four bacterial species, *M. tuberculosis, S. pneumoniae, E. coli,* and *H. influenzae* were compared. The analysis results indicated that highly conserved amino acids were largely centralized at turns 6, 7 and 8 within the LβH architecture in C-terminus, suggesting the potential active site of *M. tuberculosis* GlmU acetyltransferase. Significantly, four sequential residues N397, Y398, D399 and G400 situated within turn 8 were highly conserved, and they constituted a special insertion loop N-Y-D-G, which has been determined as a binding group of the sugar substrate in other three species [11, 12]. Additionally, the C-terminal tailing was also supposed to play a special role in the substrate binding [9]. Therefore, based on the analysis of amino acid alignment from the four species mentioned above (Fig. 1), as well as the crystallographic studies of GlmU structure of *S. pneumoniae* [12], we analyzed 10 highly conserved amino acids located in turns 6 to 10 and also in the extended tailing, probably related to the activity of GlmU acetyltransferase. Site-directed mutagenesis technology and kinetic analysis for the resulting mutant proteins were conducted to further identify the amino acid residues essential for the acetyltransferase activity of *M. tuberculosis* GlmU.

Materials and methods

Materials

Reagents related to *M. tuberculosis glmU* gene cloning and site-directed mutagenesis were purchased from TaKaRa Biotechnology (Dalian) Co., Ltd. The Ni-nitrilotriacetic acid (NTA) His-binding column from QAIGEN was used for GlmU purification. The chemicals involved in enzyme assays were obtained from Sigma-Aldrich Chemical.

Preparation of wild type *M. tuberculosis* GlmU protein

Escherichia coli BL21(DE3)/pET16b-Mtb *glmU* [6] was used to express *M. tuberculosis* GlmU protein, and the preparation of GlmU protein was carried out as described by Zhang *et al.* with a little modification [6].

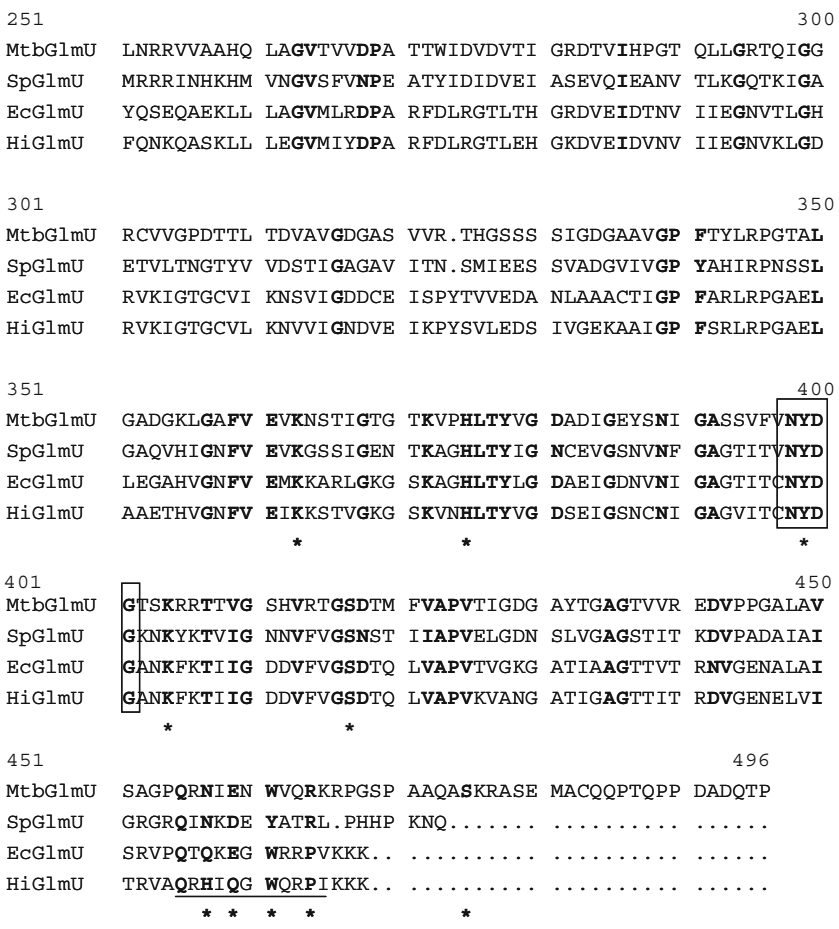

Fig. 1 Amino acid sequence alignment and analysis. GlmU acetyltransferase region (residue 251–495) of *M. tuberculosis* was aligned with its homologues from *S. pneumoniae* (SpGlmU), *E. coli* (EcGlmU) and *H. influenzae* (HiGlmU). Highly conserved residues are showed in bold. The mutational residues were determined according to the active site of *S. pneumoniae* GlmU showed in "*". The residues underlined with solid line represent the beginning part of the C-terminal extension. The residues composed of the special insert loop are signified in box

Site-directed mutagenesis of *M. tuberculosis glmU*

Site-directed mutagenesis was conducted using TaKaRa MuTanBEST Kit. Based on the DNA sequence of *M. tuberculosis glmU* gene, we designed 10 pairs of mutation-creating primers with their 5′ ends adjacent and 3′ ends in opposite directions. The pMD18-Mtb *glmU* recombinant plasmid with NdeI and XhoI sites constructed previously, was PCR-amplified in the elongation process by using high fidelity Pyrobest DNA Polymerase. The incorporation of mutation-creating primers generated different linear mutated plasmids, and these linear products were re-circled following blunting kination and self-ligation according to the instruction of TaKaRa MutanBEST Kit. Then a series of pMD18-Mtb *glmU* plasmids with point mutations were replicated in *Escherichia coli* Novablue competent cells, respectively. The selected clones were verified for the required mutations by DNA sequencing at Takara Biotechnology (Dalian) Co., Ltd.

Preparation of mutant *M. tuberculosis* GlmU proteins

The desired *M. tuberculosis glmU* mutant genes were inserted into NdeI and XhoI sites of pET16b and transformed into *E.coli* BL21(DE3) cells to express different GlmU mutants with a His-tag at N-terminus. The preparation of different GlmU mutants were carried out according to the expression protocol of wild type *M. tuberculosis* GlmU protein described previously [6]. The purified GlmU mutants were analyzed by SDS-PAGE and Western blot using (anti)-polyhistidine clone HIS-1 antibody.

Acetyltransferase assay for GlmU mutants

The acetyltransferase activity analyses of different GlmU mutants were carried out according to the previous colorimetric assay for GlmU [7]. As the enzyme assay, these purified GlmU mutants were diluted in the range of initial velocity suitable for acetylation reaction [7]. The concentrations of acetylation substrates AcCoA and GlcN-1-P were each fixed at 0.4 mM. The K_m values of different GlmU mutants for acetyltransferase were determined according to double reciprocal plot by varying five concentrations of one substrate and keeping the other one fixed (0.4 mM). The relative activities against wild type *M. tuberculosis* GlmU acetyltransferase as standard (set to 100 %) represented the changes of mutated GlmU acetyltransferase activity. The kinetic parameters of wild type *M. tuberculosis* GlmU acetyltransferase were performed as controls. All measurements were performed in triplicate to correct the trial errors and the data were analyzed by Microsoft Excel.

Results

Site-directed mutagenesis of *M. tuberculosis glmU*

According to the active site of *S. pneumoniae* GlmU acetyltransferase determined in crystallography, we selected 10 highly conserved residues within the C-terminal conserved region of *M. tuberculosis* GlmU as mutation sites, including K362, H374, Y398, K403, S416, N456, E458, W460, R463, and S474. All of these residues were substituted by Alanine for keeping the native spatial structure of *M. tuberculosis* GlmU protein (Table 1). The pMD18-Mtb *glmU* plasmid was Reverse-PCR amplified as the template using the mutation primers, resulting in different linear pMD18 with mutated *M. tuberculosis glmU* genes. After blunting kination and self-ligation, the linear mutated products were circled. All the site-specific *M. tuberculosis glmU* gene mutations were acquired successfully by sequencing analysis.

Preparation of mutant *M. tuberculosis* GlmU proteins

These site-specific *M. tuberculosis glmU* mutated genes were cloned into the NdeI and XhoI sites of pET16b vector, respectively, generating a series of pET16b-Mtb g*lmU* expression plasmids with point mutations. Each GlmU mutant protein with a fused His-tag at N-terminus was expressed in *Escherichia coli* BL21(DE3) cells and purified by Ni^{2+} affinity chromatography, respectively. SDS-PAGE and Western blotting analysis confirmed that these 10 GlmU mutant proteins were all well soluble expressed, and they were purified to nearly homogeneous by Ni-nitrilotriacetic acid (NTA) His-binding column chromatography with the apparent molecular weight of approximately 54.10 kDa similar to that of wild type *M. tuberculosis* GlmU protein (Fig. 2).

Acetyltransferase assay for GlmU mutants

The acetyltransferase activities of these 10 mutant proteins were measured using DTNB colorimetric assay established previously [7]. The results of the enzyme assay indicated that the substitution of different amino acid residues in C-terminus of *M. tuberculosis* GlmU exhibited distinct effect on the activity of GlmU acetyltransferase (Fig. 3). K362A, H374A, Y398A and W460A mutants only remained less than 10 % GlmU acetyltransferase activities. However, K403A, E458A, S416A and N456A mutants still showed 27 %, 65 %, 67 % and 86 % GlmU acetyltransferase activities left, respectively. But R463A and S474A mutants did not have any impact on catalytic activity (more than 90 % acetyltransferase activities remained).

The acetyltransferase kinetic studies of *M. tuberculosis* GlmU were performed according to the analysis of wild type

Table 1 Sequences of oligonucleotide primers used for constructing site-direct mutations of *M. tuberculosis glmU* gene

Name	Primer orientation[a]	Primer sequence[b]
K362A	F	5' GGCGCGTTCGTCGAGGTCGCCAACTCCACCATCGG 3'
	R	5' CAGCTTGCCGTCGGCGCCCAA 3'
H374A	F	5' CACCAAGGTGCCGGCCCTGACCTACGTCGG 3'
	R	5' CCGGTGCCGATGGTGGAGTTC 3'
Y398A	F	5' GCGTGTTCGTCAACGCCGACGGTACGTCCAAACG 3'
	R	5' TGGAGGCGCCGATGTTGCTGTAC 3'
K403A	F	5' AACTACGACGGTACGTCCGCCCGGCGCACCACCG 3'
	R	5'GACGAACACGCTGGAGGCGC 3'
S416A	F	5' GCACGTACGGACCGGGGCCGACACCATGTTCGTG 3'
	R	5' GAACCGACGGTGGTGCGCCGTTTG 3'
N456A	F	5' CGGCGGGTCCGCAACGCGCCATCGAGAACTGGGTG 3'
	R	5' ACACTGCCAGCGCCCCCGGCGGGACATC 3'
E458A	F	5' CCGCAACGCAACATCGCCAACTGGGTGCAGCGC 3'
	R	5' ACCCGCCGACACTGCCAG 3'
W460A	F	5' CGCAACATCGAGAACGCCGTGCAGCGCAAACGC 3'
	R	5' TTGCGGACCCGCCGACACTG 3'
R463A	F	5' AGAACTGGGTGCAGGCCAAACGCCCCGGCAG 3'
	R	5' CGATGTTGCGTTGCGGACCCG 3'
S474A	F	5' GCGGCTCAGGCCGCCAAAAGAGCCTCAG 3'
	R	5' TGGGCTGCCGGGGCGTTTG 3'

[a]F, forward; R, reverse

[b]Mutated residues are underlined

protein by double reciprocal plot, reported in Table 2. As for the K_m values against GlcN-1-P, the K_m of E458A, R463A and S474A GlmU mutants were consistent with that of wild type *M. tuberculosis* GlmU acetyltransferase; the K_m of K403A and N456A GlmU mutants were lower than that of wild type enzyme, but the K_m of S416A GlmU mutant was two-fold higher than that of wild type enzyme. As for the K_m values against AcCoA, only the K_m of S474A GlmU mutant was similar to that of wild type *M. tuberculosis* GlmU; the K_m of N456A and R463A GlmU mutants were slightly higher than that of wild type protein. In contrast, the K_m of K403A, S416A and E458A GlmU mutants were four-fold lower than that of wild type protein. However, since more than 90 % acetyltransferase activities of *M. tuberculosis* GlmU were lost due to site-direct mutagenesis, the K_m values for K362A, H374A, Y398A and W460A mutants

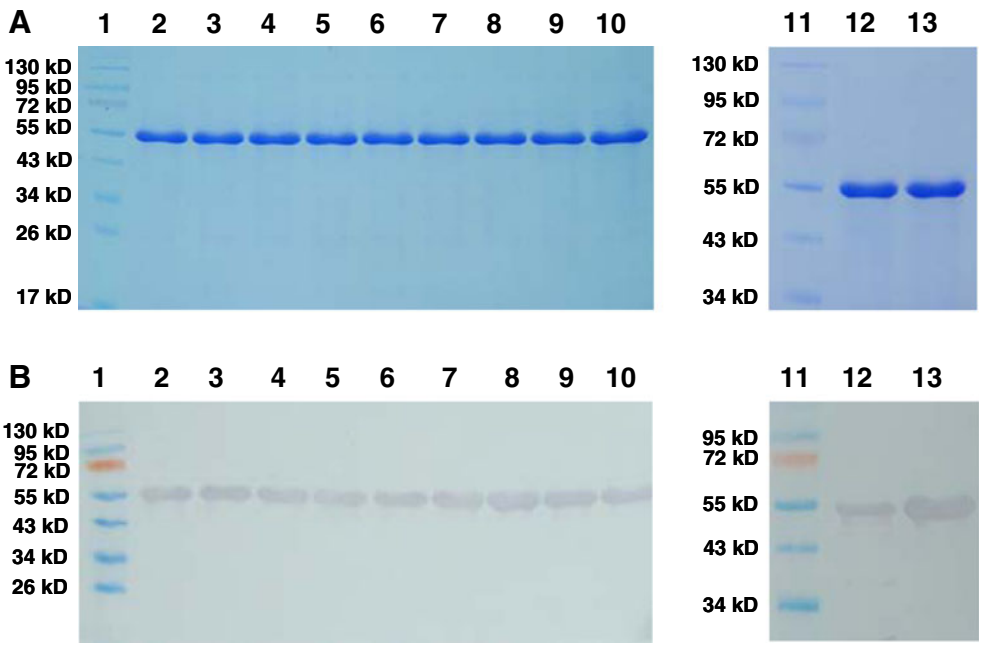

Fig. 2 SDS-PAGE and Western blotting analysis of *M. tuberculosis* GlmU mutants. Analysis of purified *M. tuberculosis* GlmU mutants by SDS-PAGE (A) and Western blotting (B). Lanes 1, 11: PageRuler prestained protein ladder (Fermentas) (in kDa); Lanes 2–10, 12–13: purified wild type GlmU, K362A, H374A, Y398A, K403A, S416A, W460A, N456A, E458A, R463A and S474A mutants, respectively

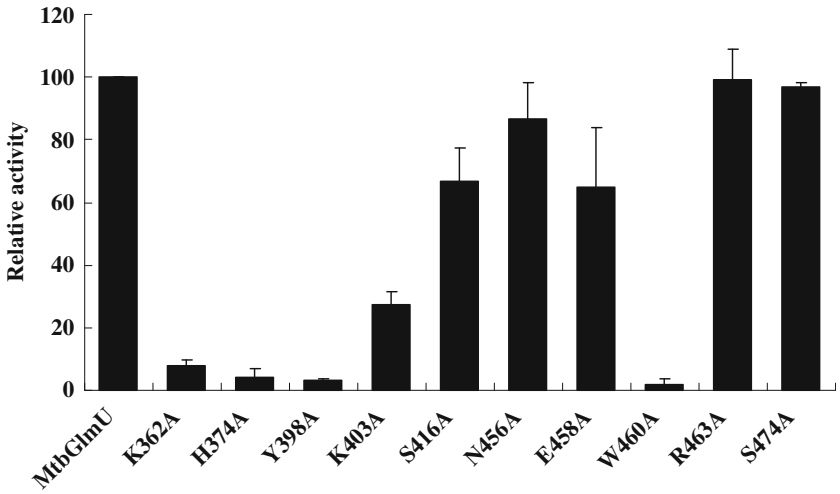

Fig. 3 Comparative acetyltransferase activity of *M. tuberculosis* GlmU mutants. The relative activities were showed by the percentage of the initial velocity of different mutants against that of the wild type GlmU protein, which was set to 100 %. Three independent experiments were carried out to calculate the standard deviation

against the two substrates were much higher than that of wild type *M. tuberculosis* GlmU (data not shown), suggesting the loss of affinity between the substrates and the enzyme.

Discussion

M. tuberculosis GlmU is a unique drug target among the enzymes involved in the formation of *M. tuberculosis* cell wall. This enzyme has two functions and catalyzes two sequential biosynthetic steps of the sugar donor UDP-GlcNAc. The GlmU uridyltransferase catalyzes the identical uridylation reaction to that in human, but has low sequence homology with acetylglucosamine-1-phosphate uridyltransferase in human. However, the reaction that GlmU acetyltransferase catalyzed is not the same as that in human. Therefore, we choose *M. tuberculosis* GlmU acetyltransferase as the target enzyme for anti-TB drug discovery. At present, high-throughput screening [13] and chemically synthesizing analogue of substrate GlcN-1-P [14] have been the effective ways to discover GlmU acetyltransferase inhibitors. However, identifying the essential amino acids of *M. tuberculosis* GlmU acetyltransferase and understanding the role they play in catalysis could be helpful to directly design inhibitors of *M. tuberculosis* GlmU acetyltransferase.

Table 2 Kinetic parameters for acetyltransferase of *M. tuberculosis* GlmU mutants

Mutant proteins	K_m GlcN-1-P (μM)	K_m AcCoA (μM)
Control (wild type)	61	224
K403A	8	56
S416A	130	70
N456A	44	343
E458A	66	60
R463A	69	304
S474A	73	242

Crystal structure of *M. tuberculosis* GlmU by X-ray diffraction displays two typical structures, Rossmann fold in N-terminus and left-handed β-helix (LβH) in C-terminus representing uridyltransferase and acetyltransferase domain respectively [8]. The active site of *M. tuberculosis* GlmU uridyltransferase is analyzed by measuring the crystal complex with different substrates or products. The crystal of *M. tuberculosis* GlmU complex with GlcNAc-1-P shows the essential residues in the active-site cavity, including Thr89, Glu166, Asn181, and Asp114, *etc.*, especially highly conserved Arg19 directly binds both of phosphates of UDP-GlcNAc [8]. However, all attempts to cocrystallize *M. tuberculosis* GlmU with acetyl-CoA, CoA or analogues are still unsuccessful [8]. The comparison of primary structures of GlmU from four species (Fig. 1) showed that the high degree of similarity in C-terminus was located between 350 and 460 residues, possibly embracing an active site of *M. tuberculosis* GlmU acetyltransferase. Some highly conserved amino acids are in line with the essential amino acids of acetyltransferase activity in *S. pneumoniae* GlmU protein [12]. Therefore, according to the active site of *S. pneumoniae* GlmU acetyltransferase, we substituted 5 highly conserved amino acids covering turn 6 (K362), turn 7 (H374), turn 8 (Y398), insertion loop (K403) and turn 9 (S416) at C-terminus to determine the active site of GlmU acetyltransferase. The other 5 substituted amino acids were located at the beginning of the extended C-terminal tailing (N456, E458, W460, R463, S474). In the LβH structure, the substitution of K362, H374, Y398 residues resulted in approximately 90 % acetyltransferase activities abolished and rather lower affinity of GlmU acetyltransferase to both substrates, demonstrating that these three amino acids were possibly located in the active site for direct substrate recognition and binding in the catalysis of *M. tuberculosis* GlmU acetyltransferase. However, the changes of K403, S416 did

not entirely inactivate GlmU acetyltransferase activity (more than 70 % and 30 % activities loss, respectively), and both mutants exhibited higher affinities to its natural substrates. It suggested that residues K403 and S416 may play an important role in converting and releasing products, whereas they could not bind the substrates directly in the catalytic process of acetyltransferase.

We predicted the secondary structures by using SWISS-MODEL Workspace to determine whether the GlmU mutants displayed conformation changes. The prediction results showed that all the mutated residues did not affect the secondary structures where the mutated residues themselves were located. However, they had effect on the secondary structures formed by the amino acids adjacent and far from those mutated residues (Table 3). For example, residue A382 was located in the β-pleated sheet in the wild type GlmU protein, while the same residue was located in a coil structure in K362A, K374A, W460A and N456A mutants. P466 was situated in the coil structure in the wild type GlmU protein, while it was involved in the helix structure in K362A, K374A, S416 and N456A mutants. It suggested that the active site of GlmU acetyltransferase was made up of both the turn structures and the extension tailing at C-terminus.

The integral C-terminal tailing of *M. tuberculosis* GlmU is 30 amino acids longer than GlmU in other species. The N-terminal region of the extended tailing still existed in other species probably reinforced its role in binding substrates due to the essentiality of conserved W460 [9]. As for W460A GlmU mutant, 97 % of the acetyltransferase activity was reduced and the affinity of W460A mutant to the two substrates was also completely undetectable. While both N456A and E458A mutants showed less GlmU acetyltransferase activity decreased than W460A mutant, but the affinity of both mutants to substrates were higher than that of wild type GlmU. The prediction results indicated that N456, E458 and W460 mutants could influence the secondary structure where residues R465-A470 and E479-Q483 were located. Whereas, W460 mutant showed more obvious changes of secondary structure at C-terminus of GlmU protein (Table 3), indicating the essentiality of the extension tailing for the catalytic process of GlmU acetyltransferase.

Therefore, we may conclude that in addition to the essential turns 6, 7 and 8, the active site also required the N-terminal region of the disordered tailing. W460 was able to embrace and bind the ligands by cooperating with K362, H374 and Y398 residues through changing conformation of the extended tailing, and then N456 and E458 as well as K403 and S416 were probably able to stabilize such active conformation for facilitating the catalytic process. It reported that S474 amino acid could stabilize the topology of the final two helices of the C terminal tailing and R463 was supposed to bind the 3' ribosyl phosphate of acetyl CoA by comparing the structures and sequences of GlmU in different bacteria [8]. However, our studies demonstrated that the substitution of S474 and R463 did not affect acetyltransferase activity and kinetic properties of *M. tuberculosis* GlmU in our study. Additionally, *M. tuberculosis* GlmU deletion (GlmU: 1–465) [9] totally lose the activity of GlmU acetyltransferase, indicating this extra 30-amino-acid of the C-terminal extension still contains catalytic sites. Further studies will be conducted to find the essential amino acid residues of the extension in C-terminus and exploring their roles in catalysis.

In summary, site-directed mutagenesis and enzyme assay of GlmU acetyltransferase determined a series of essential nature of amino acid residues, including K362A, H374A, Y398A and W460A, involved in substrate binding and catalytic regulation. However, the disordered tailing at C-terminus of *M. tuberculosis* GlmU was longer than that of the other three species (Fig. 1), and was closely related to the activity of GlmU acetyltransferase [9]. Therefore, more meticulous analyses of the extra 30-amino-acid extension by cocrystallizing *M. tuberculosis* GlmU with the substrates of acetyltransferase will be carried on for further exploring the function mechanism of this unique extension. The determination of key

Table 3 The secondary structure changes in C-terminus in *M. tuberculosis* GlmU mutants

Mutant proteins	Predicted amino acid residues related to changes of secondary structure[a]
K362A	S330 (C→E), Y342 (E→C), V359 (E→C), D382 (E→C), P466 (C→H)
H374A	D382 (E→C), N456 (E→C), I457 (E→C), P466 (C→H)
Y398A	G326 (C→E), S330 (C→E), Y342 (E→C)
K403A	G294 (C→E), S327-S329 (E→C), D333 (C→E), Y342 (E→C), S364 (E→C), D382 (E→C), A470 (C→H), E479-Q483 (H→C)
S416A	P466 (C→H), S327 (E→C)
N456A	S327-S329 (E→C), Y342 (E→C), G294 (C→E), D382 (E→C), P466 (C→H)
E458A	Q483 (H→C), S364 (E→C), N465 (H→C)
R463A	S364 (E→C), P466 (C→H)
S474A	S364 (E→C), Q483 (H→C)

[a]The secondary structure changes were predicted by SWISS-MODEL Workspace

C represents Coil structure

E represents Extended-beta

H represents Helix

amino acids of *M. tuberculosis* GlmU acetyltransferase will provide a basis for structure-guided design of inhibitors as another strategy for anti-TB drug discovery.

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