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Crystal structure of DNA polymerase III β sliding clamp from *Mycobacterium tuberculosis*

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ABSTRACT

The sliding clamp is a key component of DNA polymerase III (Pol III) required for genome replication. It is known to function with diverse DNA repair proteins and cell cycle-control proteins, making it a potential drug target. To extend our understanding of the structure/function relationship of the sliding clamp, we solved the crystal structure of the sliding clamp from *Mycobacterium tuberculosis* (*M. tuberculosis*), a human pathogen that causes most cases of tuberculosis (TB). The sliding clamp from *M. tuberculosis* forms a ring-shaped head-to-tail dimer with three domains per subunit. Each domain contains two α helices in the inner ring that lie against two β sheets in the outer ring. Previous studies have indicated that many *Escherichia coli* clamp-binding proteins have a conserved LF sequence, which is critical for binding to the hydrophobic region of the sliding clamp. Here, we analyzed the binding affinities of the *M. tuberculosis* sliding clamp and peptides derived from the α and δ subunits of Pol III, which indicated that the LF motif also plays an important role in the binding of the α and δ subunits to the sliding clamp of *M. tuberculosis*.

1. Introduction

DNA polymerase III is a multisubunit polymerase responsible for processive DNA synthesis in bacteria. *Escherichia coli* Pol III is comprised of at least 10 distinct subunits: α, ε, θ, β, τ, γ, δ, δ′, χ and ψ [1]. They are subassembled into three main parts with different functions: the polymerase core, sliding clamp and clamp loader [2,3]. The α, ε and θ subunits are contained in the Pol III core [4]; subunit α possesses the 5′-3′ polymerization activity [5], subunit ε has 3′-5′ exonuclease activity [6], and subunit θ may stabilize ε under thermal inactivation [7] and enhance the interaction of ε with α [8].

The processivity of DNA polymerase III is conferred by the β subunit (DnaN), also called the sliding clamp. It is transferred onto DNA by the ATP-dependent clamp loader complex (γ complex) of Pol III, which includes combinations of subunits γ, δ, δ′, χ and ψ [9]. When the β clamp is loaded onto the DNA, it rapidly slides along the DNA and tethers the polymerase core to the DNA for processive synthesis. The β subunit has been found to be capable of interacting with many proteins involved in DNA synthesis and repair, such as polymerase IV [10], polymerase V [11], Hda [12], MutS, DNA ligase and polymerase I [13].

Previous studies on the interactions between the sliding clamp and the clamp-binding proteins have shown that, for a number of proteins that interact with the *E. coli* β clamp, such as the Pol III α subunit, DinB, UmuC/D and MutS, the variants of a pentapeptide motif (QL[S/D]LF) played a central role [14–16]. This interaction was supported by the results from bioinformatics, yeast two-hybrid experiments and inhibition tests of the modified peptides against the interactions. Dalrymple et al. reported that the internal sequence of the α subunit (920–924: QADMF) of *E. coli* polymerase III is the binding site for α to β [14]. Furthermore, based on a crystallographic study, Jeruzalmi et al. identified that the QAMSLF sequence from the δ subunit is a β-clamp binding site [15]. Additionally, O'Donnell et al. revealed that the C-terminal sequence (1154–1160: QVELEFD) of the α subunit is another binding site for the β clamp [16].

Mycobacterium tuberculosis (*M. tuberculosis*) is a human pathogen that causes most cases of tuberculosis (TB), which is the second greatest contributor to adult mortality among infectious diseases. According to the World Health Organization's (WHO's) global TB report 2010, 1.7 million people died from TB in 2009. To reduce the burden of TB, the WHO recommends the "enable and promote research" approach as a major component of the Stop TB Strategy. To extend our understanding of the structure/function relationship of the sliding clamp and to provide a structural basis for potential drug discovery targeting TB, we cloned, purified and crystallized the β clamp from *M. tuberculosis* and solved the crystal structure using molecular replacement. In addition, we analyzed the interactions between the *M. tuberculosis* β clamp and peptides

Abbreviations: SPR, surface plasmon resonance; TB, tuberculosis; r.m.s.d., root mean square deviation.

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Table 1
Data collection, structure determination and refinement statistics.

Data collection	
Space group	$P2_1$
Cell parameters	a = 88.05 Å, b = 132.97 Å, c = 113.78 Å, and β = 109.05°
Resolution (Å)	50–2.9
Reflections (total/unique)	54139(5394)
Completeness (%)	98.2(98.7)
R_{merge}[a]	0.073(0.535)
Refinement	
R factors	
R_{cryst}[b]	0.260
R_{free}[c]	0.287
r.m.s.d. bonds (Å)	0.010
r.m.s.d. angles (°)	1.373
B factor (Å^2)	51.966
Main chain (Å^2)	50.277
Side chain (Å^2)	53.946
Ramachandran plot (excluding Pro and Gly)	
Res. in most favored regions	83.9
Res. in additionally allowed regions	16.1
Res. in generously allowed regions	0

[a] $R_{merge} = \Sigma_{hkl}\Sigma_i|I_{hkl,i}-\langle I\rangle_{hkl}|/\Sigma_{hkl}\Sigma_i I_{hkl,i}$ where I_{hkl} is the intensity of a reflection and $\langle I\rangle_{hkl}$ is the average of all observations of this reflection and its symmetry equivalents.

[b] $R_{cryst} = \Sigma||F_{obs}|-|F_{calc}|| / \Sigma|F_{obs}|$, where F_{obs} and F_{calc} are the observed and calculated structure factor amplitudes, respectively.

[c] R_{free} as R_{cryst} but calculated on 10% of the data excluded from the refinement.

from the δ and α subunits using surface plasmon resonance (SPR). Our results suggest that the conserved LF sequence also participates in the *M. tuberculosis* peptide-β clamp interaction.

2. Materials and methods

2.1. Cloning, expression and purification of the β clamp from M. tuberculosis

The *dnaN* gene encoding the β clamp from *M. tuberculosis* was cloned into pQE-30 and transformed into the *E. coli* BL21 strain. Cells from a freshly transformed clone were grown at 37 °C in the presence of 50 μg/ml of ampicillin. When the OD at 600 nm was approximately 0.6, IPTG was added to a final concentration of 0.4 mM, and the incubation was continued at 16 °C for 18 h. The cells were harvested by centrifugation, lysed by sonification and centrifuged to remove the insoluble debris. The fusion protein with a 6 × His-tag at the N-terminus was purified on a Ni–NTA column with buffer (20 mM Tris–Cl, pH 7.9, 500 mM NaCl) containing different concentrations of imidazole. The eluted protein was dialyzed against low ion buffer (20 mM Tris–Cl, pH 7.9, 20 mM NaCl) and further purified by HitrapQ and MonoQ columns successively, using a continuous gradient of 0.02–1 M NaCl in 20 mM Tris–Cl buffer, pH 7.9. The fractions from the main peak were collected, concentrated and equilibrated with gel filtration buffer (20 mM Tris–Cl, pH 7.9, 100 mM NaCl). After gel filtration purification, the protein was over 95% pure by SDS/PAGE.

2.2. Crystallization, data collection and structure determination

Crystals of β clamp were grown at 16 °C with the hanging-drop vapor diffusion method. The hanging drop was a mixture of a 1 μl protein solution (2 mg/ml) and a 1 μl reservoir solution containing 15% (w/v) PEG3350, 200 mM $MgCl_2$ and 100 mM bis-Tris at pH 5.5. The dynamic streak-seeding method was used to optimize the crystals [17], and single crystals were obtained. Initially, the crystals only diffracted to ~8 Å. Therefore, the controlled dehydration method was used to improve the crystal quality. Specifically, the equilibrated hanging drop containing the single crystals was suspended over the new reservoir solution, which contained a higher concentration of PEG3350 (25% (w/v)). After 12 h, the crystals were transferred into a new 2 μl reservoir solution for 3 min and then into the dehydrating buffer (25% (w/v) PEG3350, 200 mM $MgCl_2$, 5% (v/v) glycerol, 100 mM bis-Tris, pH 5.5) for 3 min. Finally, they were transferred into the final dehydrating buffer, which contained a higher concentration of (glycerol 10% (v/v)) for another 8 min. The obtained crystals were flash frozen in a stream of nitrogen gas and annealed on the loop. Diffraction data to 2.9 Å resolution were collected from a single crystal using synchrotron radiation at SPring-8. Data were processed and scaled with the program HKL-2000 [18]. The β clamp subunit crystallized in the monoclinic system, space group $P2_1$, with cell dimensions of a = 88.05 Å, b = 132.97 Å, c = 113.78 Å and β = 109.05°. Phases of the β clamp subunit were calculated by molecular replacement using the program Phaser [19]. The coordinates of the structure of the β subunit of *E. coli* DNA polymerase III, with sequence identity of 27%, were used as the search model (PDB code: 2POL) [20]. Coot [21] was used to manually adjust and rebuild the models. Further model refinement was carried out using Refmac5 [22] in the CCP4 [23] suite and PHENIX [24]. The refinement finally converged with R_{cryst} and R_{free} factors of 0.260 and 0.287, respectively (10% of the reflections were used for the calculation of R_{free}). Refinement statistics are listed in Table 1. The coordinates and structure factors have been deposited in the Protein Data Bank under the accession code 3P16.

2.3. SPR experiments

The interactions between peptides and the β clamp subunit were analyzed with a BIAcore 3000. The different peptides, labeled with biotin on their N-termini, and the β clamp subunit were

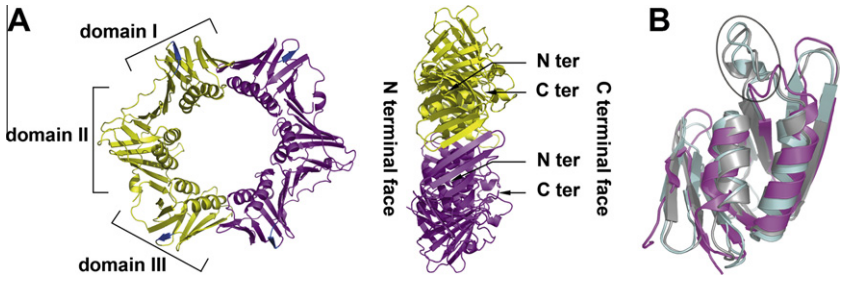

Fig. 1. Overall structure of DNA polymerase III β sliding clamp from *M. tuberculosis*. (A) Ribbon representation of the structure of the *M. tuberculosis* β clamp in the front view (left) and the side view (right), looking down the two-fold axis of the ring. The two monomers are shown in yellow and pink. Each monomer has three domains, and each domain has two α helices in the inner ring and two β sheets on the outer ring. The additional short β strands of domains I and III are indicated in blue in the front-view figure. The side-view figure shows two different faces of the β clamp. The left face, containing the N-termini of the two monomers, is the N-terminal face, whereas the C-terminal face, containing the C-termini of the two monomers, is on the right. (B) The superposition of domain I (gray), domain II (blue) and domain III (magenta). The extra α helix turns in domains I and II are circled. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

dissolved in the running buffer (20 mM Tris–Cl buffer, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.005% P20). The labeled peptides were coupled to the chip SA, a sensor harboring a carboxymethylated dextran matrix pre-immobilized with streptavidin for the immobilization of biotinylated interaction partners. The β clamp subunit (32 μM for the binding of peptides from the δ subunit and 16 μM for the binding of peptides from the α subunit) was injected for 60 s at a flow rate of 10 μl/min, and the interactions between the peptides and the β clamp subunit were monitored and presented as response units (RU) in sensorgrams.

To determine the kinetic parameters between the peptide and the β clamp subunit, different concentrations of proteins were injected into the system and flowed through the chip immobilized with the peptide, and the kinetics were analyzed by the software BIAevaluation 4.1.

3. Results and discussion

3.1. Overall structure of the β clamp from M. tuberculosis

The crystal contains three identical dimers in one asymmetric unit. Each dimer represents a closed head-to-tail ring-shaped structure, which bears a resemblance to the structure of the *E. coli* β clamp. The diameter of the whole ring is approximately 80 Å, whereas the diameter of the lumen is about 35 Å. The N-termini of the two monomers are on one face of the ring, called the

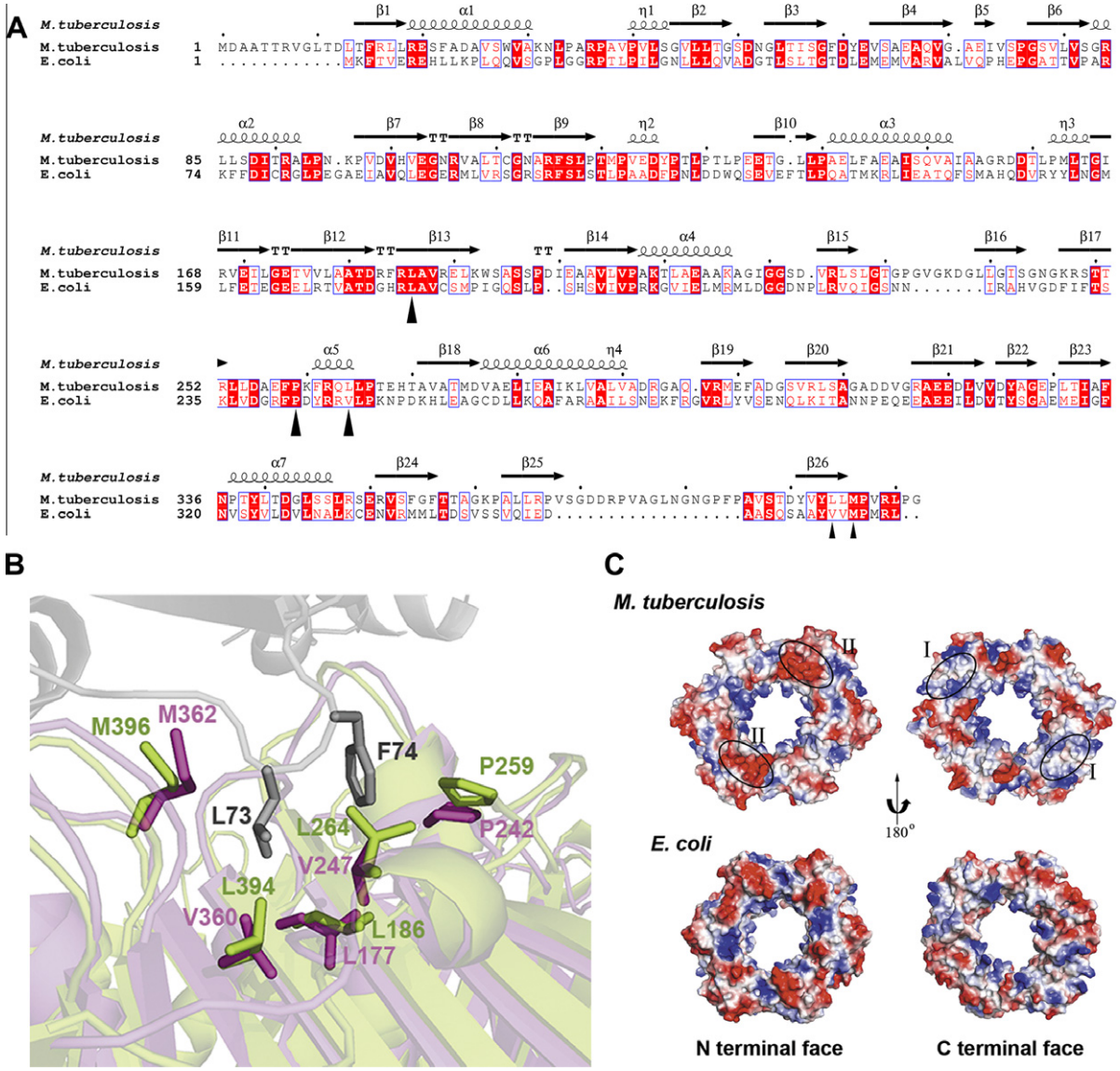

Fig. 2. Alignment of the β clamps from *M. tuberculosis* and *E. coli*. (A) Amino acid sequence alignment of β clamps from *M. tuberculosis* and *E. coli* performed by ESPript. The secondary structure elements determined from the crystal structure of the *M. tuberculosis* β clamp are labeled. Alpha helices, beta strands and strict beta turns are marked as squiggles, arrows and TT letters, respectively. White characters in red boxes show amino acids with strict identity, and red characters in the blue frame show similar amino acids. The hydrophobic residues L177, P242, V247, V360 and M362 of the *E. coli* β clamp are labeled by black triangles. These residues constitute a hydrophobic pocket, which is the main binding site for other proteins. The corresponding residues in the *M. tuberculosis* β clamp are L186, P259, L264, L394 and M396, which are also hydrophobic. (B) Overlay of the structure of the β clamp from *E. coli* and *M. tuberculosis*. The structure of the *M. tuberculosis* β clamp (green) was superimposed on the structure of the complex of the β clamp (pink) and the δ subunit (gray) of *E. coli* (PDB code: 1JQJ). (C) Distribution of the electrostatic surfaces of the β clamp from *M. tuberculosis* and *E. coli*. The regions of negative electrostatic potential are represented in red, neutral regions are represented in white, and regions of positive electrostatic potential are represented in blue. The potential distribution was generated by PyMOL [34]. The two equivalent hydrophobic pockets on the C-terminal face of the *M. tuberculosis* β clamp are circled and indicated by I. The two equivalent negative electrostatic regions on the N-terminal face, at the back of the hydrophobic pockets, are circled and indicated by II. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

N-terminal face, whereas the C-termini of the two monomers are on the other, called the C-terminal face. The two faces are not symmetrical: the C-terminal face contains more loops away from the ring. The two monomers of the homodimer are quite similar to each other. There is a noncrystallographic two-fold axis of symmetry perpendicular to the ring face. Superimposition of the Cα atoms of the two monomers reveals that the r.m.s.d. is 0.92 Å. Each monomer has three domains of similar fold containing two α helices in the inner ring and two β sheets in the outer ring (Fig. 1A).

In the *E. coli* β subunit, three domains share particular similarity, all consisting of 4 + 4 beta sheets [20]. However, in our case, there are differences among them. In domain II, one four-stranded β sheet forms a greek-key motif and the other four-stranded β sheet constitutes the N- and C-termini of the domain. In contrast, domains I and III possess an additional short β strand at the end of the first β sheet (residues 71–75 and 324–327). Although the structure of the *Streptococcus pyogenes* β clamp contains the similar extra β strands in its domain I and domain III [25], the whole structure of the *S. pyogenes* β clamp is an elliptical ring, whereas the *M. tuberculosis* β clamp adopts a circinal shape, as observed in the other β clamps.

The superposition of these three domains is shown in Fig. 1B, which reveals some unique features of the domain III relative to domains I and II. The distance between the two α helices of domain III is slightly farther than that of the α helices in the other two domains. There are extra helix turns on the C-terminal face of domains I and II, whereas the corresponding region of domain III is a loop, which is labeled in Fig. 1B.

The primary sequences of the *E. coli* and *M. tuberculosis* β clamps were aligned using ClustalW2 [26] (Fig. 2A). In the *E. coli* β clamp, a hydrophobic pocket consisting of hydrophobic residues L177, P242, V247, V360 and M362, interacts with L73 and F74 of the δ subunit (PDB code:1JQJ) [15]. The corresponding residues of the *M. tuberculosis* β clamp (L186, P259, L264, L394 and M396) form a similar hydrophobic pocket that is predominantly located in domain III (Fig. 2B), which was regarded to be the key element for interactions with other proteins.

The charge distributions of the β clamp from *M. tuberculosis* and *E. coli* were compared, and the electrostatic properties are similar (Fig. 2C). The surface of the lumen is positively charged, whereas the outer edge and the two faces are mainly negatively charged. The C-terminal face, which contains more loops away from the ring, has less negative electrostatic potential than the N-terminal face. The hydrophobic pockets on the C-terminal face are marked as region I in Fig. 2C. On the N-terminal face of the *M. tuberculosis* β clamp monomers, the region around the beginning of the last helix (region II in Fig. 2C), which is just behind the hydrophobic pocket, shows stronger negative electrostatic potential than that in the *E. coli* β clamp and might be involved in the interaction with partners.

3.2. Interaction between the *M. tuberculosis* β clamp and peptides from the δ subunit

The δ subunit is a central component of the *E. coli* clamp loader complex [27,28] that can directly associate with the β clamp. A crystallographic study of the β:δ complex from *E. coli* [15] agrees with previous biochemical data that a QAMSLF sequence containing L73 and F74 in the *E. coli* δ subunit is crucial for binding to the β clamp. Our sequence alignment of the δ subunits from *E. coli* and *M. tuberculosis* shows that the residues that correspond to the LF residues of the *E. coli* δ subunit are conserved in the *M. tuberculosis* δ subunit (L55 and F56).

To investigate the β subunit binding site of the δ subunit from *M. tuberculosis,* two peptides from the *M. tuberculosis* δ subunit were synthesized with a biotin label at their N termini: VGAYELAELLSPSLFAE (residues 42–58, peptide 1) and KVSERADFIRSEFASLR (residues 123–139, peptide 2). The affinities between the peptides and the β clamp from *M. tuberculosis* were measured by SPR. Peptide 1, including the conserved LF residues, showed strong

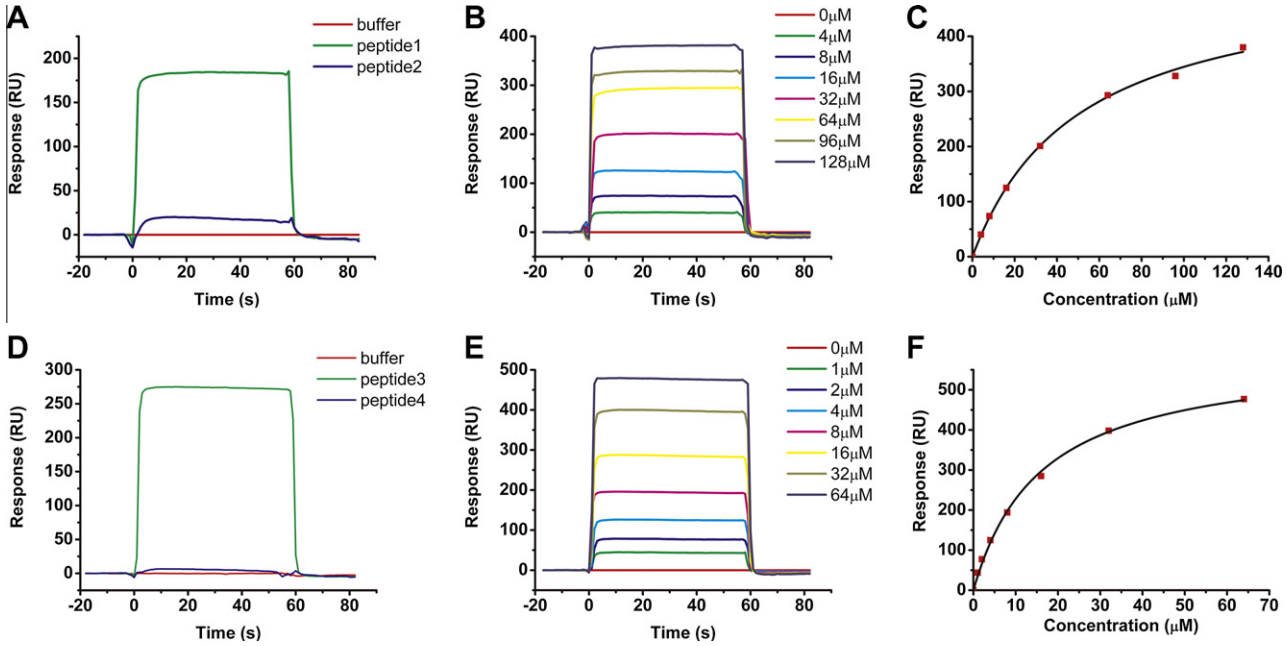

Fig. 3. Results obtained by SPR analysis of the interactions between the *M. tuberculosis* β clamp and peptides. (A) Sensorgram demonstrating the interactions between the *M. tuberculosis* β clamp and peptides 1 (green) and 2 (blue) from the δ subunit. (B) and (C) Response of peptide 1 at equilibrium vs. protein concentration. The smooth curve shows the best fit. (D) Sensorgram demonstrating the interactions between the *M. tuberculosis* β clamp and peptides 3 (green) and 4 (blue) from the α subunit. (E) and (F) Response of peptide 3 at equilibrium vs. protein concentration. The smooth curve shows the best fit. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

binding affinity to the β clamp. Peptide 2, which was a peptide from the δ subunit, showed little interaction with the β clamp (Fig. 3A). The K_D of peptide 1 and the β clamp was 50.2 μM (Fig. 3B and C). Our results show that the LF residues are essential for the interaction of the *M. tuberculosis* δ subunit with the β clamp.

3.3. Interaction between *M. tuberculosis* β clamp and peptides from the α subunit

Subunit α (Mr = 130 kDa), encoded by gene *dnaE*, is the only catalytic DNA polymerase subunit of *E. coli* Pol III [29]. The processive β clamp tethers Pol IIIα to the primer-template junction and confers processivity to the catalytic α subunit [30]. Previous studies have identified two sequences within the α subunit that are important for the interaction between the Pol III α and β subunits in *E. coli*. One sequence is at the C-terminus, including residues 1154–1160 (QVELEFD) [16]; the other is an internal site, including residues 920–924 (QADMF) [31]. When the two motifs and the oligonucleotide binding domain between them were deleted, the remaining fragment completely abolished the interaction with the β clamp [32]. The β binding affinities of 9-mer, 20-mer and 30-mer peptides, corresponding to the two motifs, were analyzed using isothermal titration calorimetry (ITC). These data suggested that the 9-mer peptide contributes the majority of the binding enthalpy [33].

The *M. tuberculosis* α subunit contains 1184 amino acids and is similar in length to the α subunit from *E. coli.* Sequence alignment of the α subunits from *E. coli* and *M. tuberculosis* showed that *M. tuberculosis* did not contain a sequence that was homologous to the *E. coli* C-terminal β clamp binding sequence, whereas residues 946–950 (QFDLF) from the *M. tuberculosis* α subunit were comparable to the residues 920–924 within the *E. coli* α subunit.

Considering that our binding assay suggested that the LF motif in the δ subunit of *M. tuberculosis* is involved in binding to the β subunit, we wondered whether an LF motif would also facilitate interactions between the α and β subunits. Therefore, we designed and synthesized two peptides with N-terminal biotin labels: GTKKAEALGQFDLFGS (residues 937–952, peptide 3); LMGDLKELL-GPGCLGS (residues 1169–1184, peptide 4), and measured their binding affinities to the *M. tuberculosis* β subunit by SPR. Similar to the interactions between the δ subunit and the β subunit, peptide 3, which consisted of the conserved LF residues, showed stronger binding affinity for the β clamp (Fig. 3D), with a K_D of 16.0 μM (Fig. 3E and F), and peptide 4, which was the C terminus of the α subunit, had little binding affinity.

Collectively, our results provide the detailed structural information of the β sliding clamp from *M. tuberculosis*, and suggest two LF-containing sequences to be the binding sites for the α subunit and δ subunit of *M. tuberculosis,* respectively. These data may be employed in the rational design of inhibitors of the β clamp from *M. tuberculosis*.

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