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Characterization of Amino Acid Residues Essential for Tetramer Formation and DNA-Binding Activity of ssDNA-Binding Protein of *Mycobacterium tuberculosis*

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Abstract—*Mycobacterium tuberculosis* Rv0054 encodes a single-stranded DNA-binding protein (MtbSSB) that is essential for survival of the human pathogen and causative agent of tuberculosis. The function of MtbSSB has been proposed to be different from its *E. coli* homolog. However, the critical amino acid residues of MtbSSB and their regulatory effects on DNA-binding ability remain to be clearly characterized. In this study, using a frequency-controlled random mutagenesis method (FRM), mutant libraries of MtbSSB were successfully constructed. On the whole, 146 single, double, and triple MtbSSB mutants, which covered 89% of the amino acid residues along the whole MtbSSB gene, were isolated. Using bacterial two-hybrid assays in combination with native PAGE assays, four new mutants, E62G, D104N, E94G/T137N, and S130P/G153N were found to totally or partially lose their ability to form tetramer. Three novel mutants, E62G, D104N, and E94G/T134N, were characterized to have a much lower ssDNA-binding activity, while one mutant, F21L, was found to have a significantly higher activity through both electrophoretic mobility shift and surface plasmon resonance assays. Interestingly, three amino acid residues, E62, D104, and E94, were found to regulate both oligomerization and ssDNA-binding activity of MtbSSB. Our work provides an important resource and should help improve the understanding of the biochemical mechanisms and structure–function relationship of the DNA-binding protein in this important human pathogen.

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Single-stranded DNA-binding protein (SSB) participates in DNA replication, repair, and recombination and is an important bioactive macromolecule in DNA metabolism [1, 2]. SSB binds specifically to single-stranded DNA (ssDNA) with high affinity, and it protects the transiently formed ssDNA from nuclease and chemical attacks and prevents secondary structure formation [1-3]. Furthermore, SSB has also been implicated in several other processes through interactions with DNA-handling enzymes such as polymerases, helicases, and nucleases [4-7]. Despite a low level of sequence identity, the three-dimensional structure of the DNA-binding domains from several species indicates a very high degree of structural conservation among tetrameric SSBs. Structural analysis of *Escherichia coli* SSB (EcoSSB) complex predicted several essential aromatic residues participating in ssDNA binding interaction, but those predictions have not been validated experimentally [8].

Mycobacterium tuberculosis, a causative pathogen for human tuberculosis (TB), infects one third of the world population and leads to almost two million deaths each year. A full understanding of *M. tuberculosis* growth and development is therefore urgently needed [9]. *Mycobacterium tuberculosis* SSB (MtbSSB), a 164-residue-long ssDNA-binding protein, can exist as a tetramer in solution with a molecular mass of 69 kDa. MtbSSB shares 84% identity with the *M. smegmatis* SSB (MsmSSB) [10]. However, MtbSSB fails to complement the Δssb strain of EcoSSB [11, 12]. Although the tertiary structures of MtbSSB and EcoSSB are similar, their quaternary structures are remarkably different. In fact, SSB is a dimer of dimers, and the EcoSSB tetramer can be obtained from MtbSSB by rotating together subunits of one of the dimers by 42° while keeping the other dimer

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unaltered. Therefore, MtbSSB has a specific structure characteristic.

In the present study, mutant libraries of the *MtbSSB* gene were successfully constructed and 146 single, double, and triple MtbSSB mutants were isolated. Their ssDNA-binding and multimer formation abilities were further investigated. Four new mutations essential for the MtbSSB tetramer formation were successfully characterized. Four mutations were found to regulate the ssDNA-binding activity of MtbSSB.

MATERIALS AND METHODS

Bacterial strains, plasmids, enzymes, and chemicals. *Escherichia coli* strain BL21(λDE3) (Novagen, USA) was used for protein expression. Plasmid pET28a (Novagen) was used to overexpress MtbSSB and its variants. Restriction enzymes, T4 ligase, modification enzymes, and Pyrobest DNA polymerase were obtained from TaKaRa Biotech (China). All reagents for frequency-controlled random mutagenesis were purchased from Sigma (USA). The reagents for bacterial two-hybrid assay were purchased from Stratagene (USA).

Preparation of MtbSSB mutant libraries. The MtbSSB mutant libraries were obtained by the modified frequency-controlled random mutagenesis (FRM) method [13]. Specifically, the wild-type *MtbSSB* gene, amplified from the genomic DNA of *M. tuberculosis* H37Rv, was purified with a DNA purification kit. Concentration of purified DNA was determined by measuring absorbance at 260 nm, and then diluted with distilled H_2O to 0.1 μg/μl. A 50-μl diluted sample was added to 5.5 μl of 3 M NaOH and incubated for 30 min at 42°C to create single-stranded DNA. Subsequently, 30 μl of 10 mM hydroquinone and 520 μl of 3 M sodium bisulfite (adjusted to pH 5.0 with NaOH), both freshly prepared, were added and mixed. The mixture was then covered with 200 μl of mineral oil and incubated at 50°C for the desired reaction time. After removing the mineral oil, the modified DNA was purified, eluted into 50 μl of distilled water, and either used immediately or stored at −20°C. The modified DNA was re-amplified by PCR using 5 μl of modified DNA as template in a 50 μl reaction system (PCR program: 3 min at 94°C followed by 10 cycles of 30 sec at 94°C, 30 sec at 40°C, and 1 min at 72°C, and finally 3 min at 72°C). The products, purified with the DNA purification kit, were ligated into vector pET-28a and then transformed into *E. coli* BL21. The resulting mutant genes were sequenced to identify the exact sites of mutation.

Expression and purification of recombinant proteins. The *MtbSSB* gene was amplified by PCR from the genomic DNA of *M. tuberculosis* H37Rv with primers: 5′-ATAAGCGGCCGCAGTGGCTGGTGACACCAC-CAT-3′ and 5′-GCCGTCTAGAGGTCAGAATGGCG-GTTCGTCATC-3′. The wild-type *MtbSSB* and its mutants were cloned into pET28a and overexpressed. *Escherichia coli* BL21(λDE3)/pET-28a-SSB was induced with 1 mM IPTG at A_{600} = 0.6 for 4 h at 37°C. Protein purification was carried out as described previously [14]. Based on purity determined by SDS-PAGE, the peak fractions were dialyzed overnight against 10 mM Tris-HCl (pH 8.0), 5% glycerol, 0.1 mM EDTA, 0.1 mM DTT, and 200 mM NaCl. Protein concentrations were determined according to a previously published procedure [14].

Electrophoretic mobility shift assay (EMSA). The following 65 bp fragment was used in the EMSA assay: 5′-CGCTCGAGTTACCATTTTCTAATATAA-TAATTAGGAGCTATCTCTTGTAATGCTTTCCAA-CTCCT-3′. The fragment was first radiolabeled at the 5′ end with [γ-^{32}P]ATP and then added to the reaction mixture. Briefly, 1 μM of the labeled ssDNA was incubated with various concentrations (1 to 4 μM) of MtbSSB in 20 mM Tris-HCl buffer, pH 8.0, 300 mM NaCl at 25°C for 30 min. Samples were loaded onto 6% nondenaturing acrylamide gels and electrophoretically separated in 0.5× TBE buffer. The gel was dried and analyzed by autoradiography to monitor MtbSSB–ssDNA complexes and free ssDNA.

Native PAGE assay. The SSB proteins and their multimer products were separated by electrophoresis on a 10% native polyacrylamide gel according to the procedures described by Zhang et al. [15]. Briefly, 40 μM SSB protein samples were mixed with 2× loading solution containing 40 mM Tris-HCl, pH 7.5, 80 mM β-mercaptoethanol, 0.05% bromophenol blue, and 8% glycerol. The protein bands were visualized by staining with Coomassie brilliant blue.

Surface plasmon resonance (SPR) analysis. The interaction between ssDNA and MtbSSB was also assayed by SPR using the BIAcore 3000 system. The 65 bp fragment was labeled with biotin and immobilized on SA chips (BIAcore). Following a period of stabilization, the purified MtbSSB and mutant proteins were passed over the chip. Experiments were performed in a running buffer (20 mM Hepes, pH 7.5, and 300 mM NaCl) at a flow rate of 10 ml/min at 25°C. Each analysis was performed in triplicate. An overlay plot was produced to depict the interaction between the two proteins.

Bacterial two-hybrid (B2H) analysis. Bacterial two-hybrid analysis was carried out according to the procedure supplied with the commercial kit (Stratagene, USA). Wild-type and mutant *MtbSSB* genes were cloned into both pBT and pTRG vectors (Stratagene). A pair of pBT-SSB and pTRG-SSB was co-transformed into the *E. coli* XR strain (Stratagene) for screening. Negative growth co-transformants, compared with wild-type *MtbSSB*, were selected on the screening medium plate containing 5 mM 3-amino-1,2,4-triazole (3-AT) (Stratagene), 8 μg/ml streptomycin, 15 μg/ml tetracy-

Table 1. Summary of mutation type and mutation frequency induced by the frequency-controlled random mutagenesis method for different treatment times

Mutagenic time[a], min	Number of GC→AT	Number of AT site mutations	Base mutation frequency[b], %	Number of amino acid mutations	Amino acid mutation frequency[c], %
20	436	39	0.32	317	0.64
40	634	41	0.45	415	0.84
60	927	23	0.64	539	1.09

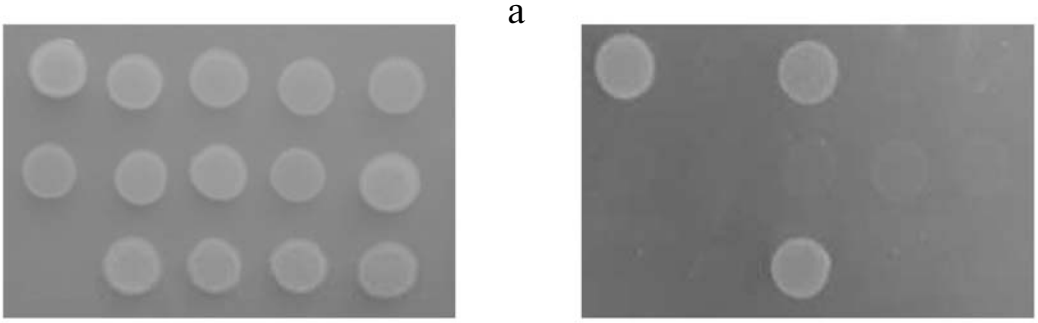
[a] A total of 300 genes were sequenced in each treatment time.
[b] Base mutation frequency was defined as the percentage of total base number divided by the number of base mutations.
[c] These data were obtained as the percentage of total base number divided by the number of amino acid mutations.

cline, 34 μg/ml chloramphenicol, and 50 μg/ml kanamycin. The plates were incubated at 30°C for 3-4 days. A co-transformant containing pBT-LGF2 and pTRG-Gal11P (Stratagene) was used as a positive control for expected growth on the screening medium. A co-transformant containing empty vectors pBT and pTRG was used as a negative control.

RESULTS

Construction of *M. tuberculosis* SSB mutant libraries. We constructed random mutant libraries of MtbSSB through the FRM method in order to identify its key amino acid residues. Three-hundred clones were selected randomly from these libraries and sequenced; 146

a



b

CK+	CK-	pBT-SSB / pTRG-SSB	pBT/ pTRG-SSB	pTRG-SSB/ pBT
pBT-F21L/ pTRG-F21L	pBT-R38W/ pTRG-R38W	pBT-E62G/ pTRG-E62G	pBT-I59T/ S80N / pTRG-I59T/S80N	pBT-E94G/ T137N / pTRG-E94G/T137N
	pBT-S130P/ G153S / pTRG-S130P/G153S	pBT-A15V/ A120V / pTRG-A15V/A120V	pBT-R56W/ T114I / pTRG-R56W/T134I	pBT-D104N/ pTRG-D104N

Fig. 1. Interaction between MtbSSB monomers assayed by bacterial two-hybrid. a) The BacterioMatch II two-hybrid system (Stratagene) was used to detect protein–protein interactions of wild-type and mutant MtbSSB monomers. Left panel: plate minus streptomycin (str) and 5 mM 3-amino-1,2,4-triazole (3-AT). Right panel: plate plus str and 5 mM 3AT. b) An outline of the plates in (a). CK+, co-transformant containing pBT-LGF2 and pTRG-Gal11P as a positive control; CK–, co-transformant containing pBT and pTRG as a negative control. Each unit represents the corresponding co-transformant on the plates.

Table 2. Constituents of the sequenced mutants

Mutagenic time, min	Single mutants	Double mutants	Triple mutants	Multi-mutants	Total mutants[a]
20	44	36	27	25	132
40	38	40	33	36	147
60	52	35	39	50	176
Total mutants[b]	134	111	99	111	455[c]

[a] Sums of total mutants in each treatment time.
[b] Sums of total mutants including different number of amino acid mutation sites.
[c] Total mutants acquired through this study.

mutants containing single, double, or triple mutations were isolated and characterized. As shown in Table 1, most of the mutations were GC→AT transitions, which is consistent with previously reported data [13]. The amino acid mutagenesis frequency for induction times of 20, 40, and 60 min was 0.64, 0.84, and 1.09%, respectively (Table 1). As shown in Table 2, 134 single mutants were obtained from all sequenced clones and together they covered 81 amino acid residues and represented about 50% of the total 164 amino acid residues of the entire *M. tuberculosis* SSB protein. In addition, double residue mutants involved 107 amino acids (65% of total), and the triple mutants involved 88 amino acids (54% of total). Taken together, 455 MtbSSB mutants were isolated. These mutations covered 146 amino acid residues of the protein and represented a coverage rate of 89%.

Characterization of amino acid residues essential for oligomerization of MtbSSB protein. We utilized the mutant libraries and performed a bacterial two-hybrid assay to characterize mutant variants that negatively affected oligomer formation of the SSB protein. Eight mutants were isolated and found to have different growth ability from wild-type MtbSSB. As shown in Fig. 1, compared with the wild-type SSB protein, the transformant strain, containing a pair of pBT-SSB and pTRG-SSB with the same mutation sites, grew very slowly or did not grow at all on the screening medium plate.

A native PAGE assay was used to further examine the effects of these eight mutations on the oligomerization of the MtbSSB protein (Table 3). As shown in Fig. 2, the wild-type protein has a major tetrameric band, showing that the native PAGE assay works well. Among the eight characterized mutants from B2H, four mutants including E62G, D104N, E94G/T137N, and S130P/G153N had bands that moved faster than wild MtbSSB protein on the gel, indicating that these mutants have lower abilities to form oligomer under the same condition. The four other mutants that we characterized did not show any significant change under this condition (Fig. 2).

In summary, four new mutants (E62G, D104N, E94G/T137N, and S130P/G153N) were successfully characterized to have a significantly lower ability in oligomer formation using both bacterial two-hybrid and native PAGE assays.

Characterization of amino acid residues involved in ssDNA-binding activity of MtbSSB. We further characterized the ssDNA-binding activities of the eight MtbSSB

Table 3. Mutants chosen for ssDNA-binding activity assays

Mutation sites of nucleic acids	Amino acid residue alteration
Mutants characterized by B2H	
61 T → C	F21L
112 C → T	R38W
185 A → G	E62G
310 G → A	D104N
166 C → T, 341 C → T	R56W, T114I
176 T → C, 239 G → A	I59T, S80N
281 A → G, 410 C → A	E94G, T137N
388 T → C, 457 G → A	S130P, G153S
Mutants predicted to affect ssDNA-binding activity	
59 G → A	R20Q
109 C → T	P37S
161 T → C	F54S
166 C → T	R56W
244 C → T	R82W
250 A → G	K84E
256 C → T	R86W
260 C → T	S87L

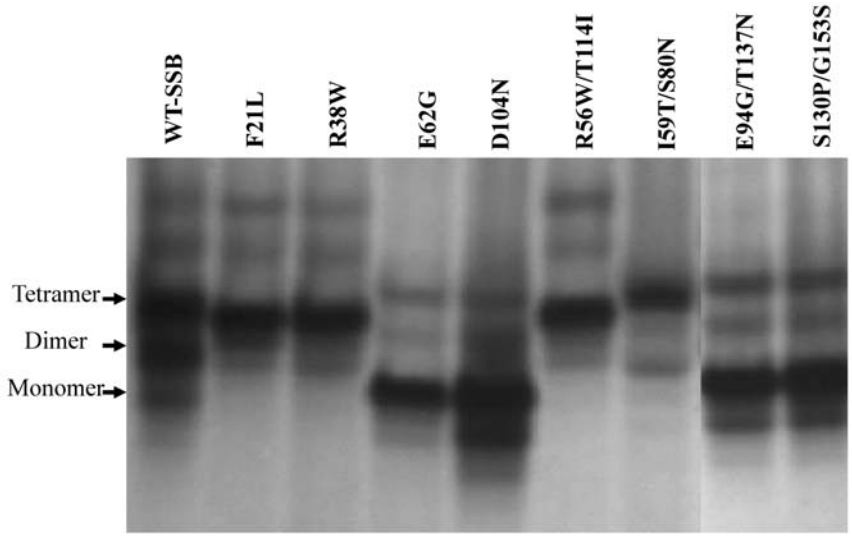


Fig. 2. Polymeric formation ability of the wild-type and mutant MtbSSB proteins assayed by native PAGE. Samples containing 40 μM wild-type and indicated mutant MtbSSB protein were added to reaction mixtures and were then separated by electrophoresis on a 10% native polyacrylamide gel.

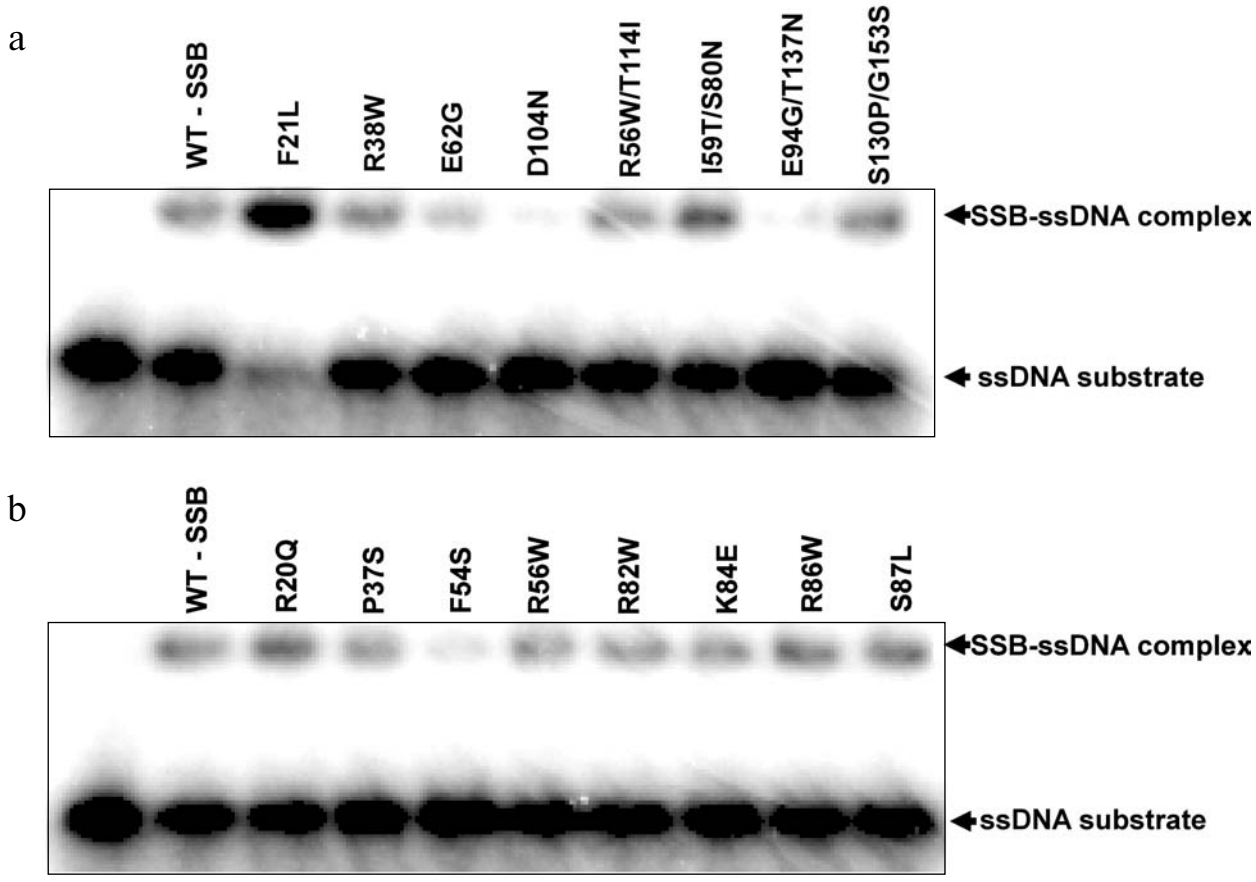


Fig. 3. Assays for the ssDNA-binding activity of mutant MtbSSBs by EMSA. Labeled 65-bp ssDNA (1 μM) was incubated with 1 μM MtbSSB or mutants in Tris-HCl buffer (20 mM, pH 8.0, and 300 mM NaCl) at 25°C for 30 min. Samples were loaded onto 6% nondenaturing acrylamide gels and separated with 0.5× TBE buffer by electrophoresis. The protein/DNA complex and DNA substrate on the gel are indicated by arrows.

mutant variants and eight other previously predicted mutants [8, 11, 16, 17] (Table 3) by two different methods. We first examined the effects using electrophoretic mobility shift assay (EMSA). As shown in Fig. 3, when 1 μM of each of these mutant proteins was added to the EMSA reaction mixture, five mutants (F21L, F54S, E62G, D104N, and E94G/T134N) were found to have significantly different DNA-binding activities compared to the wild-type protein. In particular, F21L showed an enhanced DNA-binding activity and four other mutants (F54S, E62G, D104N, and E94G/T134N) had either significantly reduced or no DNA-binding activity (Fig. 3). All of these mutants except F54S were from our mutant library (Fig. 3b and Table 3). We also confirmed the DNA-binding activities of the five mutants using concentration-course experiment (0.5, 1, 2, 3, 4 μM). As shown in Figs. 4a and 4b, the F21L mutant had a 132% DNA-binding activity relative to wild-type MtbSSB, while mutants E62G, F54S, D104N, and E94G/T134N had only 61, 40, 56.5, and 35% activity, respectively.

We further examined the effects of the five mutations on the DNA-binding activities of the protein using surface plasmon resonance (SPR) assays. When 125 nM wild-type SSB protein was passed over the 65-bp ssDNA-immobilized SA chip, a significant association response of about 150 RU (resonance units) was observed. In contrast, running buffer gave no response (Fig. 4c). A greatly decreased response (about 100 RU) was observed when 125 nM E62G, F54S, D104N, or E94G/T134N was passed over the chip (Fig. 4c). In comparison, a significantly higher response (about 600 RU) was observed when 125 nM F21L was passed over the chip (Fig. 4c). The findings of our SPR assays are thus in agreement with the results from EMSA assays described above.

Taken together, we characterized four novel mutations (E62G, D104N, E94G/T134N, and F21L) and one

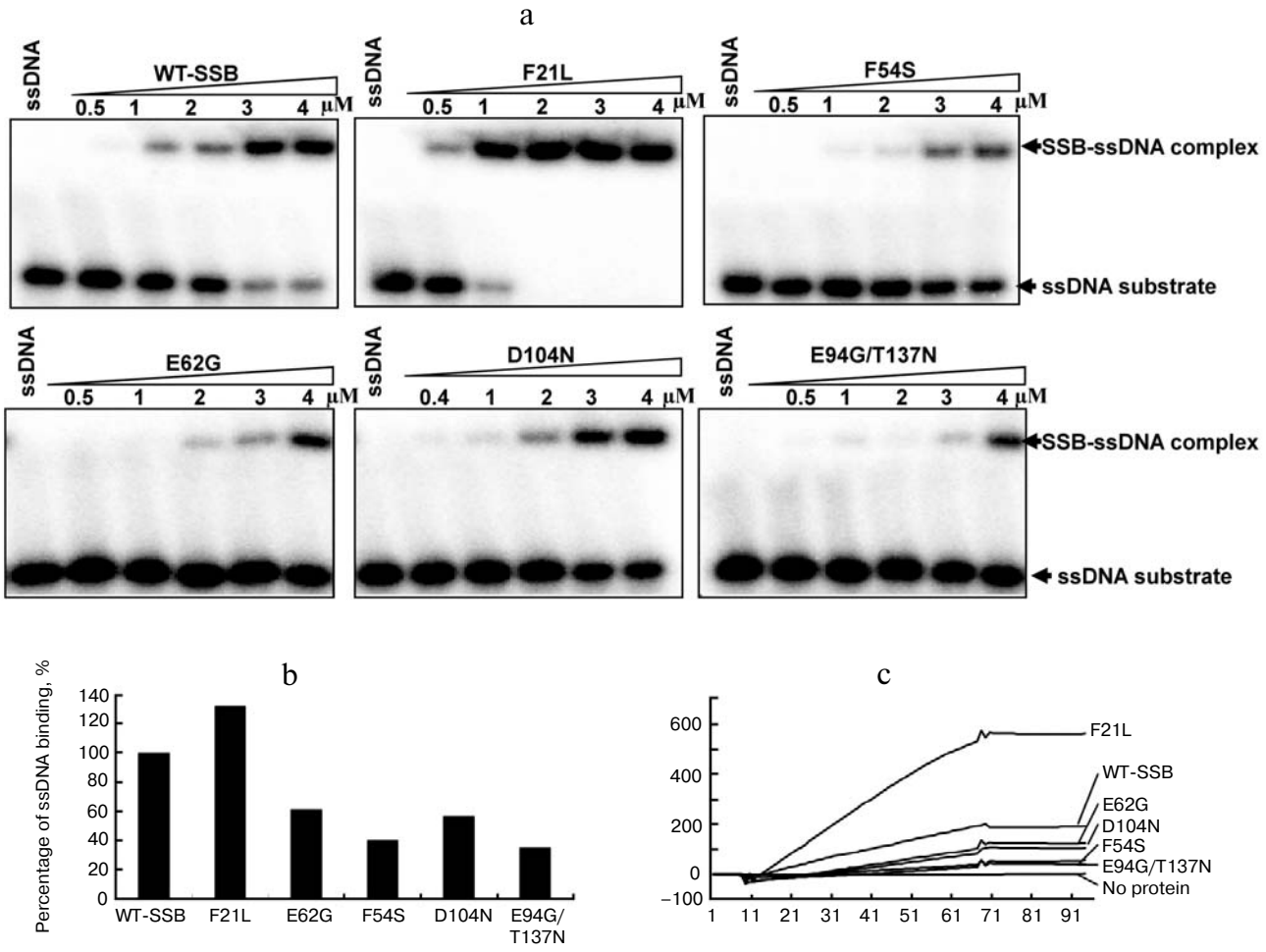


Fig. 4. a) Protein concentration course and EMSA assays for DNA-binding activity of MtbSSB. Labeled ssDNA was co-incubated with various concentrations of MtbSSB proteins (1 to 4 μM) in Tris-HCl buffer at 25°C for 30 min. Samples were loaded onto 6% nondenaturing acrylamide gels and separated with 0.5× TBE buffer by electrophoresis. b) Percentage of the shifted DNA substrate by MtbSSB. Only ssDNA-binding activities of highest concentration of each protein are shown. c) SPR assays for protein–DNA interactions. Biotin-labeled 65-bp ssDNA was immobilized on SA chips. Following a period of stabilization, purified wild-type and mutant MtbSSBs were passed over the chip. Each analysis was performed in triplicate.

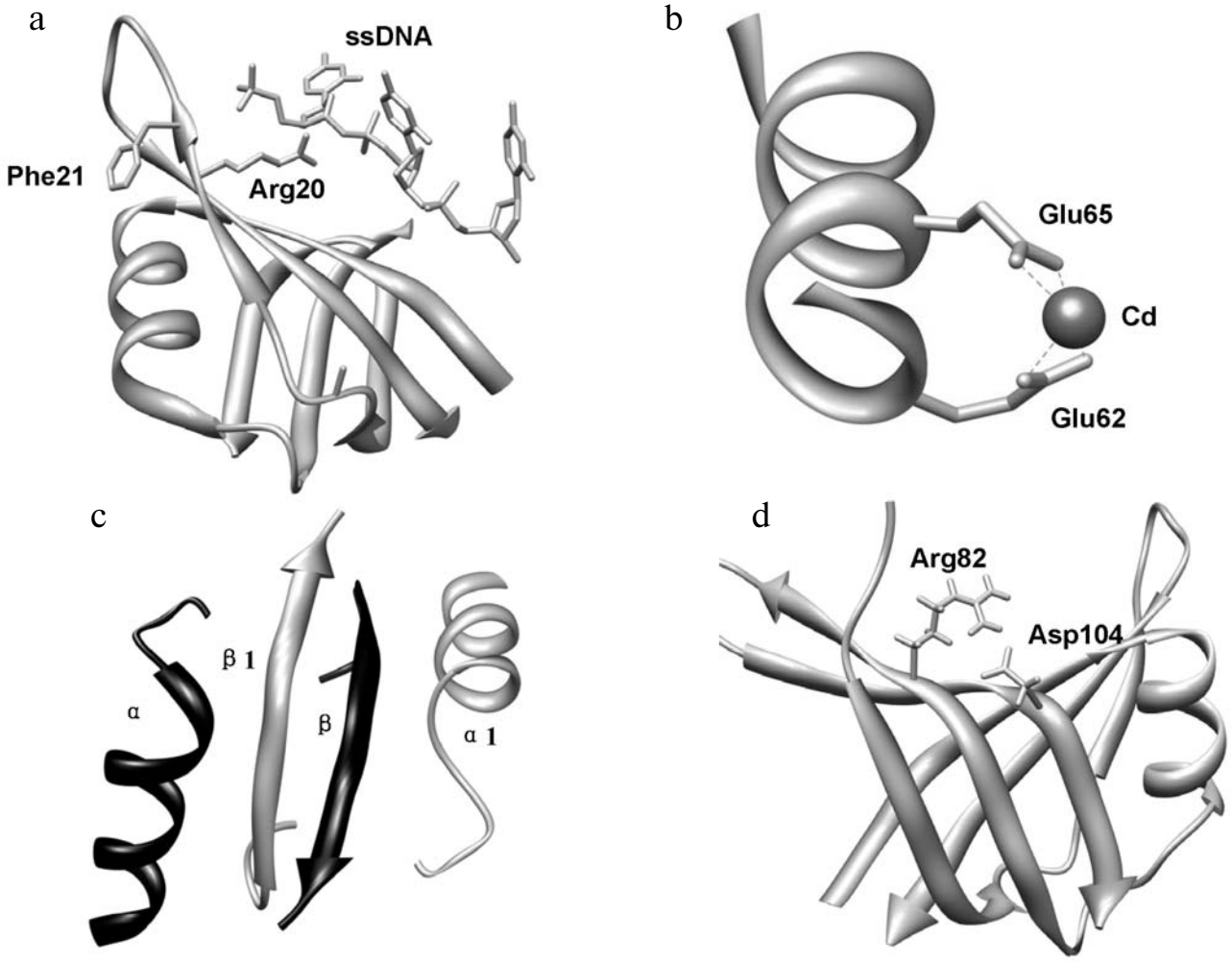


Fig. 5. A schematic diagram showing key amino acid residues of the mycobacterial SSB. a) The relationship between Phe21, Arg20, and ssDNA in MsmSSB and ssDNA. b, c) Structural characteristics of Glu62 in metal ion binding and dimer formation. d) Interaction between Asp104 and Arg82.

previously predicted mutation (F54S) that alter the ssDNA-binding activity of MtbSSB.

DISCUSSION

Like EcoSSB and *Helicobacter pylori* SSB, MtbSSB binds preferentially to ssDNA with high affinity. All three of these SSB proteins can form homotetramers and cooperatively bind with ssDNA [8, 11, 16, 17]. In the current study, the B2H assay was first designed to select MtbSSB mutants in which the interaction between its monomers was negatively affected. Based on their slow growth on selective medium compared to wild-type SSB, eight mutants were isolated and further characterized by native PAGE assay to have reduced ability to form multimer. The ssDNA-binding activity of these mutants, along with several previously predicted mutants, was further examined [8, 11, 16, 17]. We found five mutants (F21L, E62G, F54S, D104N, and E94G/T134N) in which ssDNA-binding activities of the MtbSSB were affected significantly (Fig. 3). Among them, Phe54 is an equivalent residue of Trp54 in EcoSSB, Trp68 in HmtSSB, and Phe50 in HpSSB, which are all generally acknowledged as amino acid residues essential for ssDNA binding and stabilizing the SSB–ssDNA complex in the respective proteins [8, 11, 16, 17]. In the present study, we found that the ssDNA-binding activity of MtbSSB was decreased when Phe54 was mutated to Ser, which indicates that Phe54 is a key amino acid residue for the ssDNA binding function of MtbSSB. In contrast, we found that the ssDNA-binding activity of MtbSSB increased when Phe21 was mutated to Leu. Analysis of the structure of MsmSSB bound to ssDNA has revealed that the benzene ring group of Phe21 lies away from the nucleotides of ssDNA [17]. The change of amino acid residue from Phe21 to Leu most likely makes the side-chain reside closer to the

ssDNA. In addition, this mutation would likely affect the neighboring Arg20, a basic amino acid residue, and make it rearrange and accommodate ssDNA binding (Fig. 5). Taken together, these changes may explain the increase in ssDNA-binding activity of the MtbSSB mutant F21L.

In summary, we successfully constructed mutant libraries of the *M. tuberculosis* SSB and characterized several important amino acid residues essential for its multimeric formation and ssDNA-binding activity. Taken together, our work should help further understanding of the biochemical mechanisms of action and structure–function relationship of the DNA-binding protein in this important human pathogen.

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