Molecular Dissection of Mycobacterium tuberculosis Integration Host Factor Reveals Novel Insights into the Mode of DNA Binding and Nucleoid Compaction*

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Background: mIHF belongs to a subfamily of proteins, distinct from E. coli IHF.

Results: Functionally important amino acids of mIHF and the mechanism(s) underlying DNA binding, DNA bending, and site-specific recombination are distinct from that of E. coli IHFαβ.

Conclusion: mIHF functions could contribute beyond nucleoid compaction.

Significance: Because mIHF is essential for growth, the molecular mechanisms identified here can be exploited in drug screening efforts.

The annotated whole-genome sequence of Mycobacterium tuberculosis revealed that Rv1388 (Mtihf) is likely to encode for a putative 20-kDa integration host factor (mIHF). However, very little is known about the functional properties of mIHF or the organization of the mycobacterial nucleoid. Molecular modeling of the mIHF three-dimensional structure, based on the cocrystal structure of Streptomyces coelicolor IHF domain as a bona fide relative of mIHF, revealed the proposal of Arg-171 and Arg-173, which might be important, and a conserved proline (Pro-150) in the tight turn. The phenotypic sensitivity of E. coli strains to UV and methyl methanesulfonate could be complemented with the wild-type mIHF or its alleles bearing mutations in the DNA-binding domain. Competition assays revealed that wild-type mIHF binding variants, binds with high affinity to a 30–35-bp DNA containing attB and attP sites and curve bending. The functionally important amino acid residues of mIHF and the mechanism(s) underlying its binding to DNA, DNA bending, and site-specific recombination are fundamentally different from that of E. coli IHFαβ. Furthermore, we reveal novel insights into IHF-mediated DNA compaction depending on the placement of its preferred binding sites; mIHF promotes DNA compaction into nucleoid-like or higher order filamentous structures. We therefore propose that mIHF is a distinct member of a subfamily of proteins that serve as essential cofactors in site-specific recombination and nucleoid organization and that these findings represent a significant advance in our understanding of the role(s) of nucleoid-associated proteins.

Bacterial nucleoid is a compressed helical structure composed of DNA bound to nucleoid-associated proteins, whose architecture and protein composition are regulated in a variety of bacterial species (1–3). Nucleoid-associated proteins such as DNA replication, expression through binding of the major NAPs include H-NS, HU, Fis, IHF, and StpA, which influence the topology of bound DNA by bending and bridging nonadjacent DNA segments (3). Originally discovered in E. coli as an essential host factor for integration/excision of phage λ (4), IHF links the architecture of the genome to its function inside the cell, influencing replication (5) and transcription (6–9), and serves as an integral component of several site-specific recombination systems (3, 10). E. coli IHF, a member of the DNABII structural family, is composed of two subunits, IHFα and IHFβ (~10 kDa each), each of which is required for full IHF activity (3, 8, 10, 11). E. coli IHF binds with high affinity to a 30–35-bp DNA having a conserved 3’ region with a consensus sequence WATCAANNNNTTR (where W is A or T, R is purine, and N is any base), and the 5’ region is degenerate but is typically AT-rich (8, 10, 12–14). Binding of E. coli IHF causes the DNA to adopt a U-turn, thus bringing the nonadjacent segments into close proximity (12–14). However, other studies have shown that the interaction between IHF and DNA is complex, with IHF binding to DNA via different modes that induce different DNA-bending patterns, and these DNA-binding modes are sensitive to various solution conditions (15, 16).

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3 The abbreviations used are: NAP, nucleoid-associated protein; AFM, atomic force microscopy; IPTG, isopropyl β-D-thiogalactopyranoside; mIHF, M. tuberculosis IHF; MMS, methyl methanesulfonate; PDB, Protein Data Bank; Ni2+-NTA, nickel-nitrilotriacetic acid; IPTG, isopropyl β-D-thiogalactopyranoside.
**Mycobacterium tuberculosis IHF**

Several molecular and genome-scale studies have demonstrated that the members of the IHF-HU superfamily of proteins regulate global and local gene expression in diverse species of Gram-negative and Gram-positive bacteria (17–22). Mycobacterial IHF was originally discovered in *Mycobacterium smegmatis* as a factor essential for site-specific recombination promoted by mycobacteriophage L5 integrase (henceforth called phage L5) (23, 24). Subsequently, annotation of the whole-genome sequence of *M. tuberculosis* H37Rv revealed the presence of a putative *ihf* gene in the pathogen (25). Several lines of evidence suggest that *E. coli* NAPs share relatively low amino acid identity with their counterparts in a wide variety of microorganisms, including *M. tuberculosis* (2, 3, 26, 27). For example, *M. tuberculosis* H37Rv *ihf* (*Mtihf*) is predicted to encode a single 20-kDa polypeptide compared with two different protein species in *E. coli* (25). Consequently, general features of the nucleoid structure and function described for the *E. coli* paradigm may not be relevant to other bacteria, thus emphasizing the need to understand the identity and roles of NAPs, especially in pathogenic bacteria. Furthermore, unlike wild-type *E. coli* strains, IHF is essential for the growth and viability of *M. smegmatis* and *Mycobacterium tuberculosis* (24, 28–30). However, despite these considerations, the functional properties of *M. tuberculosis* IHF (henceforth called mIHF), the mechanism underlying the formation of higher order nucleoprotein structures and compaction of DNA into nucleoid, largely remain unknown.

In this study, we address two fundamental questions regarding the identity and function of mIHF. First, is mIHF essential for bacterial growth and DNA compaction? Second, because mIHF is structurally unrelated to the IHF superfamily of proteins that serve as essential cofactors in the formation of nucleoid, the presence of a putative mIHF gene in the pathogen (25). Consequently, general features of the nucleoid structure and function described for the *E. coli* paradigm may not be relevant to other bacteria, thus emphasizing the need to understand the identity and roles of NAPs, especially in pathogenic bacteria. Furthermore, unlike wild-type *E. coli* strains, IHF is essential for the growth and viability of *M. smegmatis* (24, 28–30). However, despite these considerations, the functional properties of *M. tuberculosis* IHF (henceforth called mIHF), the mechanism underlying the formation of higher order nucleoprotein structures and compaction of DNA into nucleoid, largely remain unknown.

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**EXPERIMENTAL PROCEDURES**

**Homology Modeling and Sequence Alignment**—The full-length protein sequence of *M. tuberculosis* IHF was retrieved from Uniprot database (UniProtKB ID LOT6Q3) (31). The similarity of mIHF sequence with the experimentally determined protein structures in PDB (32) was analyzed through BLAST (33) (blast.ncbi.nlm.nih.gov). Multiple sequence alignments between different IHF proteins were obtained through T-Coffee webserver (34). The cocrystal structure of *Streptomyces coelicolor* IHF protein bound to dsDNA (sIHF; PDB code 4ITQ) was the topmost hit, sharing sequence identity of 60% (E-value 1e-20) with the C-terminal end of mIHF(87–190). Consequently, the sIHF protein, which lacks an N-terminal fragment of 86 amino acids, was chosen as the template for homology modeling using Modeler 9 version 10. The alignment between sIHF and mIHF was optimized using Promals3D (35). The secondary structures were restrained based on the boundaries derived from PSIPRED predictions (36). The refinement of the model was iteratively carried out using KoBaMin (37). The model with the lowest discrete optimized protein energy score was finally selected for analysis (38, 39).

**Western Blot Analysis**—Polyclonal anti-mIHF antibodies against purified mIHF were prepared in rabbits and characterized according to the standard procedures (40). To investigate the identity of intracellular IHF, *M. tuberculosis* H37Rv cells were cultured in Middlebrook 7H9 medium (Difco) supplemented with 10% (v/v) albumin/dextrase/catalase enrichment and 0.05% (v/v) Tween 80 in a shaker incubator with a speed of 180 rpm at 37 °C (41). Whole-cell lysates were prepared as described previously (42). Briefly, cells equivalent to 1.0 at an600 at different time points. Both were resuspended in SDS loading buffer and processed through an ultrasonic liquid processor three times for 30–40 s in the presence of protease inhibitor. Whole-cell lysates were then mixed with 1 μg of protein from the 7H9 broth and transferred onto a nitrocellulose membrane in phosphate-buffered saline. Membranes were probed in a solution containing 10 mM Tris (pH 7.4), 150 mM NaCl plus 0.1% bovine serum albumin, 0.1% ovalbumin, 0.1% Tween 20, and 0.02% sodium azide and anti-mIHF or anti-SigA antibodies for 12 h at 4 °C. The blot was subsequently washed three times with PBS and incubated for 1 h at room temperature with alkaline phosphatase- or peroxidase-conjugated (secondary antibody Sigma). Finally, the blot was developed with the Fast 5-bromo-4-chloro-3-indolyl phosphate-nitro blue tetrazolium kit (Sigma) or enhanced chemiluminescence (ECL).

**MMS and UV Sensitivity Assays**—*E. coli* wild-type or ΔihfB strains, induce DNA damage by creating site-specific recombination. Strikingly, it was discovered that functionally relevant amino acid residues and the mechanism underlying mIHF binding to DNA and site-specific recombination are different from that of *E. coli* IHFαβ. Overall, our data are consistent with the notion that mIHF is a distinct member of a subfamily of proteins that serve as essential cofactors in site-specific recombination and nucleoid organization and therefore could serve as a potential target for structure-aided drug design.
bands were excised from the gel and eluted into TE buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA).

Curved DNA and 230-bp (pB16) and 220-bp noncurved DNA (pNB10) were excised from the pB16 and pNB10 plasmids (43), respectively, by digestion with HindIII and EcoRI. DNA fragments were labeled at the 5'-end using [γ-32P]ATP and T4 polynucleotide kinase. The labeled DNA was electrophoresed through an 8% polyacrylamide gel in 45 mM Tris-broth containing 100 μM rifampicin. The wild-type strain harboring the plasmid pMtihf was grown Luria-Bertani broth supplemented with ampicillin (100 μg/ml) and chloramphenicol (100 μg/ml) at 37°C. Cells were harvested by centrifugation, washed in STE buffer (10 mM Tris-HCl (pH 8), 100 mM NaCl, and 1 mM EDTA), resuspended in buffer A (10 mM Tris-HCl (pH 8), 150 mM NaCl, and 10% (v/v) glycerol), and stored at −80°C.

Isolation of M. tuberculosis ihf Gene—The coding sequence corresponding to the M. tuberculosis H37Rv ihf (Rv1387) was PCR-amplified from the cosmid DNA (MYTC21B4) using oligonucleotides (forward primer, 5'-GAGGGCCATATGGCATGCGCACC-3' and reverse primer, 5'-CAGTGAGCTTGAACGCGCAGAGG-3') carrying restriction enzyme sites for NdeI and BamHI, respectively. PCR-amplified product was gel-purified and digested with NdeI and BamHI. The generated DNA was ligated into the DNA digested with NdeI and BamHI from the expression vector pET15b. The resultant plasmid was directionally ligated into NdeI and BamHI sites of expression vector pET15b. The resultant plasmid was designated pMtihf. The identity of the recombinant plasmid was ascertained by restriction analysis and DNA sequencing. To confirm the identity of the protein encoded by pMtihf, we raised antibodies against purified mIHF and characterized them according to the standard procedures (40).

Construction of M. tuberculosis Mutant ihf Expression Plasmids—PCR primers used for site-directed mutagenesis are listed in Table 1. The plasmid pMtihf was mutated using the QuikChange method with PfuTurbo DNA polymerase and DpnI. The arginine at position 170, 171, and 173 was replaced with alanine or aspartate. Similarly, proline at position 150 was substituted with alanine. E. coli DH5α was used for plasmid amplification. The mutations were ascertained by restriction analysis and DNA sequencing.

Expression and Purification of M. tuberculosis IHF Wild-type and Mutant Proteins—A culture of E. coli Rosetta2 (DE3)pLysS strain harboring the plasmid pMtihf was grown Luria-Bertani broth containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol at 37°C until an A600 of 0.5. The culture was incubated with gentle shaking at 37°C for 6 h. Cells were collected by centrifugation, washed in STE buffer (10 mM Tris-HCl (pH 8), 100 mM NaCl, and 1 mM EDTA), resuspended in buffer A (10 mM Tris-HCl (pH 8), 150 mM NaCl, and 10% (v/v) glycerol), and stored at −80°C. Cells were thawed and lysed by sonication (Model No. GEX-750, Ultrasonic Processor) on ice at 60% duty cycles in a pulse mode. The suspension was centrifuged in a Beckman Ti-45 rotor at 30,000 rpm for 1 h at 4°C. The supernatant was then loaded onto a Ni²⁺-NTA-agarose column equilibrated with buffer A. mIHF was eluted with a 30–500 mM linear gradient of imidazole in buffer A. Peak fractions were pooled and dialyzed against buffer B (10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM DTT, 100 μM NaCl, and 10% (v/v) glycerol). The dialyzed sample was loaded onto a double-stranded DNA-cellulose column that had been previously equilibrated with buffer B. IHF was eluted with a linear gradient of NaCl in buffer B. Peak fractions were pooled and dialyzed against buffer B. Aliquots of mIHF were analyzed by SDS-PAGE and found to be >98%. Aliquots of mIHF variants were stored at −80°C.

Expression and Purification of E. coli IHFαβ—E. coli IHFαβ was purified as described previously (44). The plasmid pET21a IHF, harboring E. coli ihfA and ihfB, was purchased from Addgene (Cambridge, MA) and transformed into E. coli Rosetta2(DE3)pLysS strain. Bacteria were grown in LB broth containing ampicillin (100 μg/ml) and chloramphenicol (34 μg/ml) at 37°C until an A600 of 0.6 and induced by the addition of 1 mM IPTG. The culture was incubated with gentle shaking at...
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37 °C for 6 h. Cells were collected by centrifugation, washed in STE buffer (10 mM Tris-HCl (pH 8), 100 mM NaCl, and 1 mM EDTA), resuspended in lysis buffer (100 mM Tris-HCl (pH 8), 1 mM EDTA, 10% sucrose, 10% glycerol, and 1 mM NaCl), and supplemented with 1 mM PMSF. Cells were lysed by sonication (Model No. GEX-750, ultrasonic processor) on ice at 60% duty cycles in a pulse mode. The suspension was centrifuged in a Beckman Ti-45 rotor at 30,000 rpm for 1 h at 4 °C. To the supernatant, (NH₄)₂SO₄ was added to 50% saturation at 4 °C, and the precipitate was removed by centrifugation. To the supernatant, (NH₄)₂SO₄ was added to 80% saturation at 4 °C, and the precipitated proteins were recovered by centrifugation. The precipitate was resuspended and dialyzed overnight against buffer A (25 mM HEPES, 0.1 mM EDTA, 5% glycerol, and 100 mM NaCl) at 4 °C. The dialysate was then loaded onto a heparin-Sepharose column that had been previously equilibrated with buffer A. IHFαβ was eluted with a 400 to 1.6 M linear gradient of NaCl in buffer A. The peak fractions were pooled and dialyzed against storage buffer B (25 mM HEPES, 0.1 mM EDTA, 10% glycerol, 100 mM NaCl). The purity of IHF was assessed by SDS-PAGE and found to be >98% pure. Aliquots of E. coli IHF were stored at −80 °C.

Microscopy—E. coli ΔihfA strain, harboring Mtihf or its variants, was grown in LB broth at 37 °C to an A₆₀₀ = 0.4. mIHF expression was induced with 0.8 mM IPTG for 2 h at 37 °C. Cells from the IPTG-induced and uninduced cultures were collected by centrifugation, washed twice with PBS, and resuspended in lysis buffer (100 mM Tris-HCl (pH 8), 1 mM EDTA, 10% sucrose, 10% glycerol, and 1M NaCl), and then mixed with 5 mM EDTA, 0.1% SDS, and 0.4 mg/ml proteinase K. Samples were incubated for 5 min at 24 °C and electrophoresed through a 0.8% agarose gel at 35 V for 10 h. Gels were stained with ethidium bromide, and the products were identified following visualization under UV light.

DNA Circularization Assay—The assay was performed as described previously (47). Reaction mixtures (20 μl) contained 20 mM Tris-HCl (pH 8.0), 150 mM KCl, 1 mM DTT, 1 mM potassium phosphate, 5% glycerol, and 0.25 nM 32P-labeled 140-bp duplex DNA (derived from digestion of pH19 plasmid DNA with Tfil) and the indicated concentrations of either mHIF or E. coli IHFαβ. After incubation at 37 °C for 30 min, 20 units of T4 DNA ligase (Fermentas) and 1× ligase buffer was added, and incubation was extended for 2 h. In reactions involving exonuclease III, samples were incubated at 37 °C for 20 min with 5 units of exonuclease III and then terminated by the addition of 1 μl of 100 mM proteinase K, followed by 1 μl of 20 mg/ml proteinase K. DNA was extracted with phenol/chloroform solution and precipitated with ethanol. The samples were resuspended in 5 μl of 6X DNA gel loading buffer. DNA was visualized using a Fuji FLA-9000 phosphorimager.

Atomic Force Microscopy—AFM experiments were performed as described previously (47). DNA fragments were prepared by digestion of closed circular DNA (pB16 and pNB10) with Scal or HindIII (34). Reaction mixtures contained 20 mM Tris-HCl (pH 7.5), 2 mM MgCl₂, 150 mM KCl, 5 ng of the indicated DNA, and IHF at 50 nM (Fig. 6B) or 200 nM (Fig. 6C), respectively. After incubation for 30 min, 5-μl aliquots were applied to the surface of freshly cleaved mica. Images were acquired using SNL (silicon-tip on nitride lever) an AFM probe (Agilent Technologies, force constant 21–98 N/m) and Agilent AFM controller operated in tapping mode in air. Imaging was done at a resolution of 512 × 512 pixels. Raw data were selected with the Picoimage software, and the same was used to “flatten” AFM images with second order polynomial fitting.

RESULTS

Sequence Alignments, Homology Modeling, and Identification of Candidate Residues for Mutagenesis—The annotated whole-genome sequence of M. tuberculosis H37Rv identified Rv1388 as the ihf gene. Multiple sequence alignment of the deduced amino acid sequence of Rv1388 revealed the presence of an additional 86 amino acids at the N terminus of mHIF but not in M. smegmatis IHF (Fig. 1A). A pairwise BLAST search of the PDB indicated that mHIF shows 60% sequence identity with S. coelicolor (sHIF) (48). Fig. 1B shows the sequence alignment between sHIF and mHIF derived from PROMALS3D (35). To generate mHIF homology model, the crystal structure of sHIF, determined at a resolution of 2.7 Å in complex with double-
stranded DNA (PDB code 4ITQ), was used as a template (48), and the homology model was determined using MODELLER (34). Fig. 1C shows the mIHF structure in complex with duplex DNA colored based upon the conservation of amino acid residues derived from alignment with unique protein entries of UniProt using ConSurf (50). Superposition of the backbone traces of the homology-modeled mIHF structure with the sIHF cocrystal structure yielded a low root mean square deviation value of 0.27 Å, indicating a good match between the two proteins. The DNA-binding site was inferred from the superposition of the cocrystal structure of sIHF-dsDNA onto the modeled structure of mIHF-dsDNA. These studies predicted an ensemble of residues in mIHF, Arg-170, Arg-171, and Arg-173, corresponding to Arg-85, Arg-86, and Arg-88 residues in sIHF (48) that might be involved in DNA binding activity. Comparison between the mIHF-dsDNA homology model and the crystal structure of E. coli IHFαβ with dsDNA revealed significant differences (Fig. 1D). The central structural element in E. coli IHFαβ is a pair of β-ribbon arms each with a critical Pro-65 residue that became inserted into the minor groove at the high affinity binding site. Additionally, IHF contacts dsDNA via the phosphodiester backbone, and the dsDNA wrapped around the protein and bent by >160° (8, 14). In mIHF, Pro-150 is the corresponding conserved residue that is embedded in a tight turn (Fig. 1C). Because it is not in contact with DNA, it is possible that it could be involved in maintaining the tertiary structure of mIHF.

**Expression Patterns of M. tuberculosis IHF at Various Growth Phases**—To assess whether the intracellular levels of mIHF alter during different stages of growth under in vitro conditions, M. tuberculosis H37Rv was grown in Middlebrook 7H9 liquid medium supplemented with 10% (v/v) albumin/dextrose/catalase enrichment and 0.05% (v/v) Tween 80 as described under “Experimental Procedures.” Growth was monitored by measuring the absorbance of cells at 600 nm (Fig. 2A). The growth transition from exponential to stationary phase began after ~140 h. To determine the levels of IHF, whole-cell lysates from cells harvested at different time points during the growth cycle were subjected to Western blot analysis using anti-mIHF-specific antibodies (Fig. 2B). In the early phases of exponential growth, the level of mIHF was low; however, its abundance steadily increased by about 4-fold when the cells reached a stationary phase of growth (Fig. 2A). Our results are in agreement with previous data on the intracellular concentration of E. coli IHFαβ, which varies from 12,000 molecules in the exponential growth phase to 55,000 molecules (~4-fold) in the stationary phase (51). Western blot analysis of M. tuberculosis SigA, which served as a loading control (52), did not reveal any significant changes in its levels during the bacterial growth cycle. The N-terminal amino acid sequence of the first 10 amino acids of mIHF indicated that indeed it has an additional 86 amino acid residues (data not shown).
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M. tuberculosis ihf Is Necessary and Sufficient for Viability of E. coli ΔihfA and ΔihfB Strains. The role of IHF in DNA binding, nucleosome compaction, and control of the cell cycle has been well established (24, 28–30). To explore the contribution of mIHF and cell viability, we investigated the ability of M. tuberculosis ihf to complement E. coli ΔihfA or ΔihfB strains for growth and against genotoxic stress. To do so, we used E. coli expression vectors in pET15b containing full-length M. tuberculosis ihf under the control of the T7 promoter. We also constructed similar plasmids in pET15b carrying Mtfh variants bearing triple point mutations in which Arg-170, Arg-171, and Arg-173 residues were replaced with Ala or Asp, predicted to be involved in DNA binding, and a plasmid-bearing point mutation in which Pro-150 was substituted with Ala (see Fig. 1). Serial dilutions of the indicated wild-type, mutant, and complemented mutant strains, treated with either UV light or MMS, were spread on LB agar plates and incubated as described under “Experimental Procedures.” Wild-type E. coli ihfA and ihfB strains (WT) showed slight or no sensitivity to the indicated doses of MMS or UV radiation (Fig. 3, A–D). Interestingly, the mIHF Arg-Ala triple mutant (R170A/R171A/R173A) was relatively more sensitive to MMS and UV radiation than Mtfh P150A in both ΔihfA and ΔihfB strains (Fig. 3, A–D). Similar results were also obtained with mIHF Arg-Asp (R170D/R171D/R173D) triple mutant against genotoxicity caused by UV and MMS in both ihfA and ihfB strains (data not shown). Immunoblot analyses indicated the presence of nearly equivalent amounts of wild-type and Arg-Arg-Ala triple mutant (R170A, R171A, R173A) mIHF proteins in ΔihfA and ΔihfB strains (Fig. 3, E and F). These results indicate that the lack of complementation in the case of Mtfh Arg-Ala triple mutant was not due to the absence of mIHF. Similar results were also seen with the expression of Mtfh Arg-Asp triple mutant and P150A proteins (data not shown). Furthermore, all the mutant proteins were stable in E. coli so that they could be purified in large amounts for biochemical and functional analyses (see below). Altogether, the data indicate that Mtfh alone is necessary and sufficient to restore viability to both ΔihfA and ΔihfB strains against genotoxic stress.

M. tuberculosis ihf Induces Compaction of DNA into Nucleoids—The observation that Mtfh helps E. coli to overcome genotoxic stress suggests that Mtfh contributes to nucleoid compaction. Analyses of uninduced M. tuberculosis and E. coli cells showed a light diffuse and even staining throughout the cell (Fig. 4a, upper panel). The noninduced M. tuberculosis ihfB strains exhibited greater sensitivity at the indicated doses of MMS or UV radiation (Fig. 3, b–d). However, ΔihfA or ΔihfB strains showed similar levels (Fig. 3 E). These data clearly establish a direct role of Mtfh and its triple mutant showed that they were expressed at similar levels (Fig. 3E). These data clearly establish a direct role for wild-type Mtfh in nucleoid organization.

Ability to Interact with attB and attP Sites Is Conserved between M. tuberculosis and E. coli IHF—The observation that the site-specific mutants failed to complement the UV and MMS sensitivity phenotypes of E. coli ΔihfA and ΔihfB strains led us to explore the mechanistic basis for their defects. Scrutinizing the sequence identity between M. tuberculosis and E. coli IHF proteins seemed insufficient, a priori, to infer the DNA binding specificity of mIHF. Accordingly, we cloned, expressed, and purified wild-type and variant forms of mIHF and E. coli IHFαβ (Fig. 5). To investigate the DNA binding properties of mIHF, we chose dsDNA containing phage L5 attB (600 bp) or attP (546 bp) sites (24, 56). Binding reactions were performed with 2 nM 32P-labeled dsDNA having attP or attB site and increasing concentrations of mIHF. Reaction mixtures were subjected to electrophoretic mobility shift assay (EMSA) as

FIGURE 2. Growth phase-dependent expression of IHF of M. tuberculosis. A, at the indicated time points of M. tuberculosis H37Rv growth, cells were harvested, and whole-cell lysates were prepared. B, 50 μg of protein from whole-cell lysates at each time point of growth were separated by SDS-PAGE and transferred onto the nitrocellulose membrane. The blots were probed with anti-mIHF or SigA-specific antibodies followed by secondary antibodies. Protein signals were visualized following Western blotting as described under “Experimental Procedures.” SigA protein served as an internal loading control.
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M. tuberculosis IHF Binds with Greater Affinity to Curved DNA, attP and attB than Noncurved DNA—A striking feature of *E. coli* IHFαβ is its ability to bind phage λ attB and attP sites and induce bends to facilitate the formation of higher order structures (8, 10, 14). *M. smegmatis* IHF appears to promote the integration of phage L5 in a fashion similar to that of *E. coli* IHFαβ (24, 28). To gain further insights into mIHF DNA binding activity, we chose to determine the binding affinity of mIHF to 230-bp curved DNA (derived from plasmid pB16) and 220-bp noncurved dsDNA (derived from plasmid pNB10) (43). The curved DNA is characterized by a high (78%) A + T content (43). Binding reactions were performed as described above. We found that mIHF bound with higher affinity to curved dsDNA over noncurved dsDNA substrate (compare Fig. 7, A with E). Furthermore, relatively lower protein concentration of mIHF was required to form complexes with curved DNA than with noncurved DNA. Quantitative analysis indicated that the affinity of mIHF for curved DNA is 2-fold higher compared with noncurved DNA (Fig. 8E).
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![Image of Mycobacterium tuberculosis IHF](image_url)

**FIGURE 4.** mIHF induces the nucleoid compaction. Confocal laser scanning microscopy images of DAPI-stained M. tuberculosis DHB4 (a) and E. coli ΔihfA mutant strain with Mtihf (b) and E. coli ΔihfA mutant strain with Mtihf variant (R170A R171A R173A) (c) from uninduced and 0.8 mM IPTG-induced cultures. a, brightfield images. b, representative pictures depicting the three-dimensional reconstruction of images. c, confocal laser scanning microscopy images of uninduced (uninduced) and 0.8 mM IPTG-induced (induced) M. tuberculosis DHB4 cells harboring Mtihf. Nucleoid stained with DAPI. The false-red color corresponds to the cell body. c, confocal laser scanning microscopy images of DAPI-stained E. coli ΔihfA cells harboring Mtihf or Mtihf variant (R170A/R171A/R173A) from uninduced and 0.8 mM IPTG-induced cultures.

Binding of mIHF, but Not E. coli IHFaβ, to Phage L5 attP and attB DNA Produces a Salt-stable Complex—Elucidation of the thermodynamic parameters of protein-DNA interactions is crucial to the understanding of factors that dictate the function of protein-DNA complexes. The binding affinity and specificity of E. coli IHFaβ depend on the salt concentration (58, 59). At low salt concentrations below 100 mM, the specificity of IHF is low, and the specificity increases as the salt concentration increases, up to 250 mM. We reasoned that subtle differences between mIHF and E. coli IHFaβ in their affinity to dsDNA substrates were not fully pronounced in the above assay. We therefore investigated the stability of protein-DNA complexes formed by mIHF and E. coli IHFaβ in the presence of increasing salt concentrations. Interestingly, even 1.5 M NaCl was not sufficient to dissociate the complexes formed with attP or attB containing DNA by mIHF (Fig. 10). However, complete dissociation of complexes formed by E. coli IHFaβ with the same substrates occurred in the presence of 150 mM NaCl (Fig. 10). Next, we examined the stability of complexes formed by mIHF or E. coli IHFaβ with curved and noncurved DNA. The complexes formed by the wild-type mIHF remained stable, even in the presence of 1.5 M NaCl. In contrast, the stability of complexes formed by E. coli IHFaβ with noncurved DNA decreased at a salt concentration of 200 to 300 mM NaCl. These results were obtained in the case of NaCl on the effect of NaCl on the stability of mIHF-attP and mIHF-curved DNA complexes formed with noncurved DNA. Among the potential explanations for this finding, one possibility is that binding to attP and attB results in encircling of DNA, perhaps by protein-protein interactions, hence the establishment of a salt-stable complex. This possibility was supported by the observation that binding of mIHF to curved DNA results in the formation of nucleoid-like structures (see below).

**M. tuberculosis IHF Mutants Lacking DNA Binding Activity as Well as E. coli IHF Fail to Stimulate Site-specific Recombination Promoted by Phage L5 Integrase**—One of the hallmarks of IHF family of proteins is their ability to serve as essential cofactors in the integration of phage genome into or excision from the host chromosome. Previous studies have shown that M. smegmatis IHF is essential for recombination promoted by phage L5 integrase (23, 28). To further suggest biological significance, we sought to examine the ability of mIHF and its variants to stimulate site-specific recombination as described previously (28, 56). In this assay, recombination between phage L5 attP and attB sites generates a new DNA species with molecular weight of ~8 kb. As shown in Fig. 11A, recombination promoted by phage L5 integrase was stimulated by wild-type mIHF in a concentration-dependent manner. In contrast to wild-type mIHF, Arg to Ala triple mIHF mutants failed to stimulate recombination, even at higher concentrations (Fig. 11B). Similar results were obtained in the case of Arg to Asp triple mIHF mutant protein (data not shown). As shown above (Fig. 3), although P150A mIHF mutant partially complemented the UV and MMS sensitivity of E. coli ΔihfA and Δihfb strains (Fig. 3), the mutant protein failed to form a stable complex with DNA (Fig. 6). We reasoned that the P150A mIHF-DNA complex might not be sufficiently stable to persist during EMSA. Given

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this scenario, we examined the ability of the P150A mIHF variant to stimulate integrase-promoted recombination. Interestingly, the P150A mutant protein was active and stimulated site-specific recombination, albeit at somewhat higher concentrations than the wild-type mIHF (Fig. 11C). The product formed by P150A was identical to that formed by wild-type mIHF. Restriction analysis of the recombination products ascertained that the new species of DNA is the product of site-specific recombination between attP and attB sites (data not shown). Next, we asked whether a heterologous IHF could stimulate site-specific recombination promoted by phage L5 integrase. As shown in Fig. 11D, E. coli IHFαβ failed to stimulate the reaction. The failure is perhaps due to the inability of E. coli IHFαβ to interact with phage L5 integrase to form a functional intasome (60). The combined data demonstrate that mIHF is structurally (at the primary and three-dimensional level) and functionally distinct from E. coli IHFαβ.

M. tuberculosis IHF Promotes Bending of Duplex DNA—It is known that DNA architectural proteins, such as E. coli IHFαβ, have the capacity to drastically modify DNA structure by bending (3). To investigate whether mIHF has the ability to promote DNA bending, we used T4 DNA ligase-catalyzed circularization of 140-bp DNA fragment excised from pUC19 plasmid DNA, which was devoid of intrinsically bent sequence. The principle underlying this assay is based on the fact that bending of DNA would bring the two ends into close proximity to facilitate ligation. In this assay, we used E. coli IHFαβ as a positive control. We incubated the 32P-labeled DNA fragment first with increasing concentrations of E. coli IHFαβ or mIHF followed by T4 DNA ligase. Subsequently, reaction mixtures were treated with exonuclease III to remove linear DNA molecules. The DNA products in the reaction mixture were separated by PAGE and visualized by phosphorimaging of the dried gels. In the presence of E. coli IHFαβ, and in agreement with previous studies (3, 10), we observed the formation covalently closed circular DNA, which was resistant to exonuclease III digestion (Fig. 12A). This species was absent where ligation was performed in the absence of E. coli IHFαβ (Fig. 12A, lane 2). We next examined the ability of mIHF to generate covalently closed DNA molecules. We found that mIHF, over the same range of concentrations, not only exhibited the ability to generate covalently closed DNA molecules but also had the capacity to bridge DNA
molecules into linear multimers, albeit to a much lesser extent (Fig. 12B, lanes 4–8). We considered that the formation of a linear multimer seen with mIHF posits a role in the overall organization and compactness of the nucleoid.

**Different Modes of mIHF Binding to DNA—** A number of studies have shown that E. coli IHF/H9251/H9252 binds with high affinity to 30–35 bp having a conserved 3′ region with a consensus sequence **W**ATCAA**N**NNN**R** (where **W** is A or T, **R** is purine, and **N** is any base), and the 5′ region is degenerate but is typically AT-rich (8, 10, 12–14). To gain further insights into the structural features of mIHF-DNA complexes, we generated DNA fragments that had **A**T tracts embedded either in the center or near the end (Fig. 13A). Reactions were performed under conditions similar to those used for mobility shift assays. The products of the reactions were visualized using AFM. In the absence of mIHF, dsDNA fragments were devoid of bent structures and tangles (Fig. 13B, panels i and v). Reactions performed with DNA fragments containing **A**T tract at the center and limiting amounts of mIHF, we observed that mIHF binding was predominantly at the center so that the two arms extended to either side of the complex (90%, n = 120) (Fig. 13B, panels ii–iv). Furthermore, this mode of binding resulted in sharp bends around the protein core, and the estimated contour lengths were in agreement with mIHF binding at the center (Fig. 13B, panels ii–iv). Under identical conditions, with a DNA fragment containing an **A**T tract near the end, binding
was primarily at the ends with no bending (Fig. 13 B, panels vi–viii). Interestingly, we observed globular structures at the ends, indicating the formation of higher order nucleoprotein structures. A further increase in the amount of mIHF led to the formation of two distinct types of structures, one with the DNA fragment having A\textsubscript{T}11001 tracts near the end; mIHF first bound to the end and then to the end lacking the A\textsubscript{T}11001-tract (n = 160) (Fig. 13 C, panels i and ii). However, for DNA fragment that had A tracts embedded at the center, mIHF binding resulted in the formation of nucleoid-like structures in which DNA is fully wrapped around the protein core (Fig. 13 C, panels iii–iv). The two modes of mIHF binding have important implications for the compaction of DNA into nucleoids and the formation of higher order nucleoprotein structures.

### DISCUSSION

A great deal of structural and biochemical work has been devoted to understanding the functional properties of E. coli IHF\textsubscript{αβ}. Very little is known about the components of nucleoid, and their function in mycobacteria, but such knowledge is crucial for understanding of the changes that M. tuberculosis nucleoid experiences in response to the frequently altering milieu of the host. Multiple sequence alignment and homology modeling of the mIHF three-dimensional structure indicated that it is unrelated in sequence and structure to the prototype E. coli IHF\textsubscript{αβ}, but it is similar to that of sIHF (Fig. 1). Notwithstanding the structural differences, mIHF alone was necessary and sufficient for the restoration of viability in both E. coli \textit{ihfA} and \textit{ihfB} strains against genotoxic stress, and it could induce DNA compaction and catalyze site-specific recombination. The discovery that functionally relevant amino acid residues and the mechanism that governs mIHF binding to DNA as well as DNA bending are different from that of both E. coli IHF\textsubscript{αβ} and sIHF, and provides protection from genotoxic stress, is striking. Overall, our data are consistent with the notion that mIHF is a distinct member of the family of proteins that serve as essential cofactors in site-specific recombination.

### TABLE 2

Dissociation constants for IHF-DNA complexes

| DNA substrates                  | K\textsubscript{d} values (\textmu M) |
|---------------------------------|--------------------------------------|
| Curved DNA (pB16)               | 0.60 ± 0.010                        |
| Noncurved DNA (pNB10)           | 0.90 ± 0.015                        |
| attP site-containing substrate  | 0.56 ± 0.012                        |
| attB site-containing substrate  | 0.53 ± 0.005                        |

[FIGURE 8. Binding of E. coli IHF\textsubscript{αβ} to double-stranded DNA containing phage L5 attB and attP sites as well as curved and noncurved DNA. Assay was performed as described under “Experimental Procedures.” Reaction mixtures contained 2 nM \textsuperscript{32}P-labeled attB (A) or attP (B), curved (C), and noncurved (D) duplex DNA in the absence (lane 1) or presence of 0.2, 0.3, 0.4, 0.5, 0.6, 0.75, 1, 1.2, and 1.5 \textmu M E. coli IHF\textsubscript{αβ} (lanes 2–10), respectively. The open triangle on the top of the gel denotes increasing concentrations of E. coli IHF\textsubscript{αβ}. E, graphical representation showing the extent of binding of E. coli IHF\textsubscript{αβ} to DNA containing attB and attP sites as well as curved and noncurved DNA.

There is a table with the following header:

| DNA substrates                  | K\textsubscript{d} values (\textmu M) |
|---------------------------------|--------------------------------------|
| Curved DNA (pB16)               | 0.60 ± 0.010                        |
| Noncurved DNA (pNB10)           | 0.90 ± 0.015                        |
| attP site-containing substrate  | 0.56 ± 0.012                        |
| attB site-containing substrate  | 0.53 ± 0.005                        |

Two modes of mIHF binding have important implications for the compaction of DNA into nucleoids and the formation of higher order nucleoprotein structures.

### DISCUSSION

A great deal of structural and biochemical work has been devoted to understanding the functional properties of E. coli IHF\textsubscript{αβ}. Very little is known about the components of nucleoid, and their function in mycobacteria, but such knowledge is crucial for understanding of the changes that M. tuberculosis nucleoid experiences in response to the frequently altering milieu of the host. Multiple sequence alignment and homology modeling of the mIHF three-dimensional structure indicated that it is unrelated in sequence and structure to the prototype E. coli IHF\textsubscript{αβ}, but it is similar to that of sIHF (Fig. 1). Notwithstanding the structural differences, mIHF alone was necessary and sufficient for the restoration of viability in both E. coli \textit{ihfA} and \textit{ihfB} strains against genotoxic stress, and it could induce DNA compaction and catalyze site-specific recombination. The discovery that functionally relevant amino acid residues and the mechanism that governs mIHF binding to DNA as well as DNA bending are different from that of both E. coli IHF\textsubscript{αβ} and sIHF, and provides protection from genotoxic stress, is striking. Overall, our data are consistent with the notion that mIHF is a distinct member of the family of proteins that serve as essential cofactors in site-specific recombination.
and nucleoid organization. Furthermore, we believe that our results represent an important contribution and provide insights into those functions of mIHF that are likely to be essential for growth and viability of \textit{M. tuberculosis}.

Studies of different bacterial systems have revealed the existence of complexities in the number and intracellular concentrations of NAPs (1–3). In many bacteria, the intracellular levels of IHF are dependent on the growth conditions and influence the patterns of gene expression in a temporal fashion (2, 3). Very little is known on the expression patterns and biological significance of NAPs in mycobacteria. Here, we show that the expression of mIHF follows a dynamic pattern as a function of the growth cycle (Fig. 2). Like \textit{E. coli} IHFαβ (3), expression of mIHF manifests during the early exponential phase, increases during the mid-exponential phase, and then reaches a plateau in stationary phase. The growth phenotypes of \textit{E. coli} mutants lacking both IHF and HU are more severe than the phenotypes of single mutants (53–55). However, it remains possible that IHF mutants may display mild growth attenuation that is not severe enough to be identified in the experiments described in the literature. In contrast to \textit{E. coli}, null mutations of \textit{ihf} in both \textit{M. tuberculosis} and \textit{M. smegmatis} are lethal (24, 28–30). Therefore, we used \textit{E. coli} \textit{ΔihfA} and \textit{ΔihfB} strains to explore the biological roles of mIHF under certain stringent environmental conditions. Strikingly, we found that Mtihf alone could complement the growth and MMS and UV sensitivity phenotypes of \textit{E. coli} \textit{ΔihfA} and \textit{ΔihfB} strains as well as induce DNA compaction, repair, and replication function as a barrier in the defense against genotoxic stress and/or in the regulation of multiple DNA repair pathways.

Our comprehensive biochemical analyses suggest that wild-type mIHF, its mutant variants, binds \textit{attB} and \textit{attP} sites, forms stable nucleoprotein complexes, and stimulates site-specific recombination. Furthermore, by using molecular modeling efforts on the biological and biochemical properties of mIHF, we found that the alleles affecting DNA binding failed to complement \textit{E. coli} \textit{ΔihfA} and \textit{ΔihfB} strains, and the variant proteins lacked detectable DNA binding activity. Molecular modeling studies showed that the Pro-150 in mIHF is not in contact with DNA and therefore is unlikely to be essential for mIHF DNA binding activity. Intriguingly, we found that the P150A mutant protein failed to display significant DNA binding activity as assessed by EMSA. We speculated that the inability of Pro-150 mutant to bind DNA could be due to the instability of DNA-protein complex during gel mobility assays. This premise is supported by the observation that the Pro-150 mutant protein was able to catalyze site-specific recombination under solution conditions. The similarity in sequence and structure of mIHF and sIHF raises the question of how these two closely related proteins perform their functions with requirements for binding specificity. Although mIHF can bind nonspecific sequences, it forms especially “stable” high affinity complexes with cognate DNA that are not disrupted by the addition of high salt and competitor DNA. However, sIHF interacts nonspecifically with double-stranded DNA with \( K_{d} \) in the micromolar range (48). The ability to interact with cognate DNA as well as nonspecific sequences at varying affinities is a common characteristic of the IHF-HU DNABII superfamily of proteins (3, 8, 10). In the co-crystal structure of sIHF with 19-bp DNA, the interface between sIHF and DNA is less extensive and showed no significant DNA bending (48). Although the amino acid residues of sIHF involved in DNA binding have not been identified, it is devoid of the conserved proline residue present.
FIGURE 11. **Wild-type mIHF is an essential cofactor for site-specific recombination reaction promoted by phage L5 integrase between attB and attP sites.** Assay was performed as described under “Experimental Procedures.” **A,** wild-type mIHF; **B,** Arg to Ala triple mutant (R170A, R171A and R173A); **C,** P150A mIHF mutant. **D,** *E. coli* IHFαβ. **Lane 1,** DNA markers; **lane 2,** dsDNA containing attB site; **lane 3,** negatively supercoiled plasmid DNA containing attB site; **lanes 4–13,** reaction performed with 0.5 μg of phage L5 integrase and 0.05, 0.1, 0.25, 0.5, 0.75, 1, 1.5, 2, and 2.5 μg of wild-type mIHF, Arg to Ala triple mutant (R170A/R171A/R173A) or P150A mIHF variants or *E. coli* IHFαβ, respectively. The assays were conducted at least three times for each of the proteins with typical results depicted in A–D.

FIGURE 12. **E. coli IHFαβ and mIHF promote the circularization of 140-bp DNA fragment.** The assay was performed as described under “Experimental Procedures.” **A** and **B,** reaction mixtures containing 0.25 nM 32P-labeled 140-bp DNA was incubated in the absence (lanes 1, 2, and 9) or presence of 20, 40, 80, 160, and 200 nM mIHF (lanes 4–8), units U of T4-DNA ligase. **Lane 3** in A and B, DNA was incubated with 200 nM *E. coli* IHFαβ or mIHF but no ligase. **Lanes 9–12,** reaction mixtures incubated with Exo III, after T4 ligase. The assays were conducted at least three times for each of the proteins with typical results depicted in A and B.
in *E. coli* IHF that becomes inserted into the minor groove and introduces DNA bends (8). However, the lack of DNA bending in the cocrystal structure of sIHF-DNA may not be due to lack of proline, possibly due to short stretch of duplex DNA.

Our studies reveal that mIHF causes wrapping and bending of short DNA fragments, suggesting a probable mechanism underlying the formation of nucleoid-like structures. By analyzing both target and nontarget DNA substrates, we observed that mIHF displays relatively higher affinity for phage L5 attP and attB sites and DNA rich in A/T sequences. These findings are in agreement with earlier studies for *E. coli* IHFαβ (10, 12, 13, 61, 62). Comparison of over 170 known *E. coli* IHFαβ-binding sites has led to the identification of a consensus DNA-binding motif (10, 62, 63). Among these, the two most highly conserved elements are the sequence WATCAA starting near the center of the site and the second sequence TTR located 4 bp in the 3′ direction from WATCAA (62–64). Other high affinity binding sites of *E. coli* IHFαβ contain poly(A)-tract containing 4–6 adenines (65, 66). Although mIHF exhibits relatively high affinity for phage L5 attP and attB sites, further studies are necessary to identify the consensus binding motif(s) in these sites. Since its discovery (4), *E. coli* IHFαβ has attracted considerable attention because of its role in various DNA transactions, wherein IHF-induced sharp bends or DNA loops coordinate the cooperative assembly of multicomponent nucleoprotein complexes (2, 3, 8). The biochemical data, combined with the crystal structures, have shown that *E. coli* IHFαβ binds to the minor groove of DNA and bends the double helix by >160° (14, 67, 68). Other studies have shown that *E. coli* IHF αβ binding to DNA, and subsequently DNA bending, occurs in a concerted fashion (67–69).

Nucleoids are highly conserved, bifunctional molecular entities that control DNA architecture in the cell (98, 99). DNA bending and packaging of DNA are both key roles in the regulation of transcription by modulating the state of DNA structure (1–3). An array of mechanisms has been described by which NAPs mediate compaction of DNA into nucleoids and regulate gene expression. As the affinity of NAPs to DNA is generally weak, cooperative binding is therefore essential to increase the local concentrations of NAPs near the target sites. Other mechanisms include introduction and constraining of DNA supercoiling and bridging of adjacent segments of DNA. Collectively, these processes are thought to influence the gene order along the genome and likely to regulate the temporal order of gene expression (1–3). Despite these advances, our knowledge with respect to the factors and the molecular mechanism that determines the choice between the architectural and regulatory roles of NAPs remains poorly understood. Our study provides insights into the determinants involved in the formation of filamentous and nucleoid-like structures. Altogether, our findings are consistent with the notion that the dual roles by IHF may be determined by its binding position relative to the genes it controls.

While considering the capacity of mIHF to engage in genome organization and as a global transcriptional silencer, it is useful to understand its physical impact on the DNA to which it binds. Our study suggests that the sequence determinants and their context might influence the choice between the above-mentioned processes. We also note that this is a fundamental mechanism pertinent to nucleoid organization and gene expression, which may be generally applicable to all the IHF-regulated genes and binding sites in bacterial cells (70). Given the fact that

![Figure 13](image-url)
sIHF and mIHF are structurally related, interestingly, sIHF is not essential for growth but is required for normal chromosome compaction (48). However, because IHF is essential for growth of M. tuberculosis, it is likely to be involved in multiple essential biological functions as well as in the maintenance of genome integrity; as such, this can be exploited in drug screening efforts.

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