MukE and MukF Form Two Distinct High Affinity Complexes

Received for publication, February 16, 2007 Published, JBC Papers in Press, March 12, 2007, DOI 10.1074/jbc.M701402200

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The MukBFE complex is essential for chromosome segregation and condensation in Escherichia coli. MukB is functionally related to the structural maintenance of chromosomes (SMC) proteins. Similar to SMCs, MukB requires accessory proteins (MukE and MukF) to form a functional complex for DNA segregation. MukF is a member of the kleisin family, which includes proteins that commonly mediate the interaction between SMCs and other accessory proteins, suggesting that the similarities between the MukBFE and the SMC complexes extend beyond MukB. Although SMCs have been carefully studied, little is known about the roles of their accessory components. In the present work, we characterize the oligomeric states of MukE and MukF using size exclusion chromatography and analytical ultracentrifugation. MukE self-associates to form dimers \((K_D \approx 18 \pm 3 \mu M)\), which in turn interact with the MukF dimer to form two distinct high affinity complexes having 2:2 and 2:4 stoichiometries (F:E). Intermediate complexes are not found, and thus we propose that the equilibrium between these two complexes determines the formation of a functional MukBFE with stoichiometry 2:2:2.

Structural Maintenance of Chromosomes (SMC) proteins participate in most genetic transactions affecting chromatin structure. SMCs have a characteristic domain arrangement. The N- and C-terminal regions joined by a long helix associate to form a bipartite ATPase, the head domain. A hinge domain in the middle of the protein disrupts the central helix and reverses its orientation, creating a long anti-parallel coiled-coil region (1, 2). Heterodimerization of SMC through the hinge domain confers the characteristic V-shaped architecture (3). The head domains can further interact either intra- or intermolecularly to form higher ordered structures (4). By association with accessory proteins SMCs form specialized complexes for chromosomal condensation, sister chromatid cohesion, or DNA repair (5–8). One of the accessory proteins is normally a kleisin that contains at least one winged helix domain and mediates the interaction of SMCs with additional accessory proteins (9).

Prokaryotes encode a single SMC that forms a homodimer, facilitating the study of its effects on DNA topology (3, 4, 10). Bacillus subtilis SMC associates with two accessory proteins, ScpA and ScpB. ScpA bridges the interaction between BscMC and ScpB, although both ScpA and ScpB are kleisins (11, 12). Curiously, a subset of proteobacteria including Escherichia coli does not have SMC homologues. Instead, they encode three proteins essential for chromosome partitioning, MukB, MukE, and MukF, (13–15). Defects in the muk genes cause abnormal localization of nucleoids, anucleate cell formation, temperature-sensitive colony formation, and hypersensitivity to novobiocin (14, 16). MukB has been classified as a distant relative of the SMC proteins because of its molecular architecture and the phenotypes associated with mukB mutant strains. MukF contains a winged helix domain and bridges the interaction between MukB and MukE, and thus it has been classified as a kleisin (17).

Overproduction of MukB causes DNA condensation to an extent similar to that observed when the MukBFE complex is overexpressed, but it does not reduce the production of anucleate cells or rescue the temperature sensitivity of MukBFE-deficient cells (18). Thus, MukE and MukF seem to participate in maintenance of chromatin architecture rather than chromosome condensation (18). Indeed, electron microscopy studies of MukB and MukBFE show that the MukE and MukF proteins mediate the formation of higher ordered structures that could work as chromatin scaffold (19). MukE forms a stable complex with MukF (20), but the stoichiometry and the biological relevance of such complex is still unclear. In the present work, we characterize the oligomerization states of MukE, MukF, and their complexes using size exclusion chromatography and analytical ultracentrifugation. MukF is a dimer in solution, whereas MukE exhibits reversible monomer–dimer equilibrium. MukE and MukF form two high affinity complexes with stoichiometries F2E2 and F2E4. Co-expression of MukE and MukF yields a roughly equimolar mixture of the two complexes, suggesting that both these complexes are present in vivo.

EXPERIMENTAL PROCEDURES

Protein Purification—MukE (pAG8009), MukF (pAG8011), and MukFE (pAG8135) were purified similarly. Cell pellets were resuspended in buffer A (20 mM Tris-HCl, pH 8, 0.5 mM NaCl, 1.4 mM β-mercaptoethanol, 5% (v/v) glycerol) containing protease inhibitors and lysed by sonication. Cleared lysates were loaded onto a HiTrap nickel-chelating column (GE Healthcare), washed with buffer A containing 45 mM imidazole,
and eluted with 0.3 M imidazole. Proteins were further purified over a MonoQ column (GE Healthcare). Fractions containing pure protein were concentrated and stored at 4°C in 20 mM Tris-HCl, pH 8, 0.1 M NaCl, 1 mM EDTA, 1.4 mM β-mercaptoethanol, 5% (v/v) glycerol. All subsequent experiments were conducted in storage buffer.

Size Exclusion Chromatography—Three sets of experiments were conducted at MukF concentrations corresponding to 4, 1, and 0.5 mg/ml. MukF was mixed with increasing amounts of MukE to obtain samples at stoichiometries of 2:1, 2:2, 2:4, 2:6, and 2:8 (F:E). Each sample was incubated 15 min on ice, spun down, and loaded onto a Superdex-200 column (GE Healthcare) equilibrated with storage buffer.

Sedimentation Equilibrium—Experiments were conducted at 4°C on a Beckman Optima XL-A analytical ultracentrifuge. Samples of MukE and MukF were studied at loading A280 ranging from 0.43 to 1.2 and rotor speeds of 6, 8, 10, 12, 14, and 16 krpm. Data were acquired using 6-hole cells as an average of four absorbance measurements at 280 nm using a radial spacing of 0.001 cm. Sedimentation equilibrium was achieved within 48 h. Data collected at different speeds and different loading concentrations were analyzed simultaneously in terms of various species analysis models using SEDPHAT.4.1b (21) to obtain the sample molecular mass. In the case of MukE, data collected at six loading concentrations were analyzed globally in terms of a reversible monomer-dimer self-association model using mass conservation in SEDPHAT 4.1b. Solution densities ρ were measured at 20°C on a Mettler-Toledo DE51 density meter and corrected to 4°C. The partial specific volume υ used for subsequent calculations were 0.7336 cm^3 g^−1 (MukE) and 0.7341 cm^3 g^−1 (MukF). These values were calculated based on the amino acid composition of the proteins using SEDNTERP (www.jphilo.mailway.com). To characterize the complexes formed between MukF and MukE, similar experiments were performed for 2:1, 2:2, 2:4, 2:6, and 2:8 loading mol ratios (F:E). In each case three loading A280 of ~0.4, 0.8, and 1.2 were studied and analyzed globally. The selected loading ratios of 2:1, 2:2, and 2:4 were also studied at a loading A230 of ~0.4 with data collected at 230 nm.

Sedimentation Velocity—Experiments were conducted at 4°C on a Beckman Optima XL-A analytical ultracentrifuge. Samples of MukF (loading volume of 300 μl) were studied at loading A280 of ~1.0 and rotor speeds of 50 and 55 krpm. Samples of MukE were analyzed in a similar fashion at loading A280 of 0.29 to 1.0 as well as at a loading A230 of 0.8. Loading ratios of F:E ranging from 2:1 to 2:8 were studied at loading A280 of ~1.0 and rotor speeds of 45, 50, and 55 krpm. Aluminum double centerpiece cells were used. 100–125 scans were acquired at approximately 3-min intervals as a single absorbance measurement at 280 nm and a radial spacing of 0.003 cm. Data were analyzed using SEDFIT 9.2 (22) in terms of a non-interacting discrete species for F2 and F2E4 or in terms of a continuous c(s) distribution. Sedimentation coefficients s were corrected to s20,w based on the solvent density and viscosity (η) calculated based on the composition in SEDNTERP. Sedimentation velocity profiles for selected F:E mixtures were also analyzed in terms of a hybrid local continuous/global discrete model in SEDPHAT. In these analyses three species corresponding to F2, F2E, and an unknown intermediate were considered. The masses and experimental sedimentation coefficients for the F2 and F2E4 species were fixed. Sedimentation velocity data obtained for MukE at loading concentrations of 15, 32, and 50 μM were each analyzed in SEDPHAT 4.1b in terms of reversible monomer-dimer equilibrium. Mass conservation constraints were implemented.

Hydrodynamic Calculation—The sedimentation coefficients s obtained were used to calculate the frictional coefficient f, using the relation s = M(1 − υp)/Nf, where M is the molecular mass, υ the partial specific volume, p the solution density, and N Avogadro’s number. The value of f was compared with the smallest possible frictional coefficient f0 for a sphere of equivalent mass (4): f0 = 6πηr = 6πη(3 Mv/4πN)^1/3 where r is the radius of the sphere and η the solution viscosity.

RESULTS

Oligomerization of MukF and MukE—Purified MukF eluted as a single peak from a Superdex-200 column earlier than expected for a protein of 50,579 Da (Fig. 1A). Based on published data (17, 20), the early elution time of MukF is expected due to dimerization and its non-globular asymmetric shape. The size exclusion chromatography profile showed some tailing of the MukF peak toward smaller molecular masses, suggesting either the equilibrium between various species or a non-ideal interaction of MukF with the column resin. To determine whether MukF existed exclusively as dimer, we carried out sedimentation equilibrium experiments at three loading concentrations using the exact same protein preparation as for the size exclusion chromatography experiments (Fig. 1B and supplemental Fig. S1). A global analysis in terms of single ideal solute returned excellent fits with an experimental molecular mass of 105 ± 3 kDa, confirming that MukF is a monodisperse dimer (n = 2.07 ± 0.06). The monodispersity of the MukF dimer was further confirmed by sedimentation velocity experiments and continuous c(s) analysis (Fig. 1C). Data analysis in terms of a single species yielded a corrected s20,w of 4.85 ± 0.05 S with excellent fits. Based on these data, an f/f0 ratio of 1.6 was determined, indicative of an elongated shape and consistent with the anomalous migration noted by size exclusion chromatography.

MukE (25,894 Da) also eluted earlier than expected from a Superdex-200 column at a time point corresponding to an apparent molecular mass of ~44 kDa (Fig. 1A). The elution time and the marked asymmetry of the peak were indicative of a monomer-dimer self-association. To further characterize this interaction, we carried out sedimentation equilibrium experiments at six loading concentrations (15–56 μM, Fig. 1D and supplemental Fig. S2). The weight-average molecular mass increased with the loading concentration, and like the size exclusion chromatography profiles, the molecular masses obtained were larger than those expected for a monomer but smaller than those expected for a dimer. A simultaneous data analysis in terms of reversible monomer-dimer equilibrium returned a log10 Kd value of 4.74 ± 0.06 with excellent fits, which corresponds to a Kd of 18 ± 3 μM. Attempts to model the sedimentation equilibrium data in terms of other self-association models led to poorer fits. Sedimentation velocity experiments on MukE at various loading concentrations also sup-
ported a reversible monomer-dimer equilibrium, based on the continuous c(s) profiles observed (Fig. 1, C and E). We therefore analyzed the sedimentation velocity data collected at 280 nm in terms of a single ideal solute (see supplemental Fig. 51 for the rest of the data used in the global analysis). Best fits, corresponding to a molecular mass of 105 ± 3 kDa, are shown as black lines through the experimental points. The corresponding distributions of the residuals are also shown. C, continuous c(s) distributions on sedimentation velocity data collected at 55 krpm for MukF (blue) and MukE (red) at loading concentrations of 14 and 52 μM, respectively. D, sedimentation equilibrium profiles for MukE at 56 μM shown in terms of A_280 versus the radius R for data collected at 4.0 °C and 8 (orange), 10 (yellow), 12 (green), 14 (cyan), and 16 (brown) krpm. These data, along with data collected at 15, 20, 25, and 38 μM MukE, were analyzed globally in terms of a reversible monomer-dimer self-association (see supplemental Fig. 52 for the rest of the data used in the global analysis). Best fits, corresponding to a K_d of 15 ± 3 μM, are shown as black lines through the experimental points. The corresponding distributions of the residuals are also shown. E, continuous c(s) distributions on sedimentation velocity data collected at 55 krpm for MukE at loading concentrations of 6.8 μM (A_230 = 0.80, pink), 15 μM (A_280 = 0.29, red), and 50 μM (A_280 = 0.95, brown). The dashed gray lines at 2.3 and 3.6 S show the sedimentation coefficients determined in SEDPHAT for the MukE monomer and dimer, respectively.

MukE Forms Two High Affinity Complexes with MukF—Complexes at different ratios of MukF (10 μM) and MukE were prepared and fractionated by size exclusion chromatography. The elution profiles observed at ratios of 2:1 and 2:2 (F:E) showed progressive disappearance of free MukF in favor of two larger molecular mass species consistent with complex formation (Fig. 2A, top panel). Samples at 2:4 ratios showed a single peak, and thus we assumed that MukF and MukE form a stable complex having a 2:4 stoichiometry (F2E4). Indeed, samples at ratios of 2:6 and 2:8 showed the F2E4 peak and an additional peak corresponding to excess MukE (Fig. 2A, bottom panel).
Sedimentation velocity experiments at a 2:4 (F:E) loading ratio resulted in a single species with a corrected $s_{20,w}$ of 7.40/110060.05 S and an $f/f_0$ of 1.7, as evidenced by the continuous c(s) distribution (Fig. 2B). Based on $f/f_0$, the asymmetric shape observed for both MukF and MukE is retained upon complex formation. Sedimentation equilibrium studies of this sample were consistent with the presence of a single species having a mass of 206/110067 kDa, confirming the formation of the F2E4 complex (supplemental Fig. S3). Further analyses carried out at lower loading concentrations, with data collected at 230 nm, yield similar results ($M = 201 \pm 4$ kDa, $n = 0.98 \pm 0.02$) (Fig. 2C), showing that MukF and MukE interact with submicromolar affinities.

Interestingly, sedimentation velocity experiments carried out at 2:2 and 2:1 (F:E) loading ratios indicated that F2E4 was present along with free MukF and an intermediate complex having a sedimentation coefficient of 6.3 S (Fig. 2B, top panel). These distributions and the relative abundance of each species are virtually identical to the size exclusion chromatography profiles (Fig. 2, A and B). An analysis of the sedimentation velocity profiles in terms of a global discrete model containing only three species was carried out. In this model the masses and sedimentation coefficients of the F2 and F2E4 species were fixed. Excellent data fits were observed with a third species having a sedimentation coefficient of 6.25 ± 0.05 S and an estimated molecular mass of 135 ± 10 kDa (data not shown). Sedimentation equilibrium experiments returned weight average molecular masses of 189 ± 5 and 177 ± 5 kDa for the complexes formed in the 2:2 and 2:1 mixtures, respectively. If the 6.25 S species represents an F2E2 complex, then the weight average molecular masses obtained by sedimentation equilibrium are in agreement with those based on the integration of the sedimentation velocity c(s) profiles (Fig. 2B and data not shown). This intermediate complex is therefore proposed to have a 2:2 (F:E) stoichiometry representing the binding of a single MukE dimer to a dimer of MukF. In very good agreement with the size exclu-

![FIGURE 2.](image-url)
sion chromatography data, sedimentation velocity experiments carried out at 2:6 and 2:8 (F:E) loading ratios indicate that the F2E4 complex was present along with excess MukE (Fig. 2, A and B), demonstrating that a MukF dimer binds a maximum of two MukE dimers.

To analyze whether the two MukE binding sites on the MukF dimer are independent, a series of MukF:MukE mixtures (4:1 to 2:4) were analyzed by sedimentation velocity. When plotted as a function of $y = [\text{MukE}]/2[\text{MukF}]$, the mole fractions of F2, F2E2, and F2E4, obtained by integration of the c(s) distributions, closely tracked the functions $(1-y)^2$, $2y(1-y)$, and $y^2$, respectively (Fig. 2D), demonstrating that two MukE dimer binding sites on the MukF dimer are independent and do not show any form of cooperativity. These observations provide further confirmation for the F2E2 stoichiometry of the intermediate complex.

The F2E4 and F2E2 Complexes Form in Vivo—In parallel, mukE and mukF were subcloned into the same vector with a 6× histidine tag at the N terminus of MukE. Co-expressed MukFE was purified over nickel-chelating, MonoQ and Superdex-200 columns. This three-step purification protocol yielded pure complex as judged by SDS-PAGE (Fig. 3A). However, the asymmetry of the peak eluting from the sizing column suggested the presence of multiple unresolved species (Fig. 3B). Sedimentation velocity experiments (loading $A_{280} = 1.1$) revealed the presence of mainly two species corresponding to the F2E2 (2.2 μM) and the F2E4 (2.9 μM) complexes, along with traces of free MukF (0.32 μM) (Fig. 3C). These concentrations correspond to mole fractions of 0.06, 0.406, and 0.535 for the F2, F2E2 and F2E4, respectively, and a MukE:MukF ratio of 1.45. Sedimentation equilibrium experiments returned a weight average molecular mass of 186 ± 5 kDa consistent with the presence of mainly F2E2 and F2E4 complexes in nearly equimolar amounts.

DISCUSSION

We have shown that MukF exists exclusively as a monodisperse dimer in the concentration range studied, whereas MukE shows a reversible monomer–dimer equilibrium, characterized by a $K_d$ of 15–18 μM. Purified MukF and MukE form two stable complexes with stoichiometries of 2:2 and 2:4, respectively. The F2E2 and F2E4 complexes are also found in samples purified from bacteria transformed with a co-expression plasmid, reflecting that both can be formed in vivo at least when the two proteins are overexpressed. Our data do not support stoichiometries of F2E1 or F2E3 (Fig. 2, C and D), suggesting that only the dimeric form of MukE interacts with MukF. Loading ratios of 2:4 (F:E) returned exclusively the F2E4 complex, whereas loading ratios of 2:2 (F:E) yielded both F2E2 and F2E4 complexes along with free MukF (Fig. 2, A and B), suggesting that F2E4 forms with a higher affinity than F2E2. Indeed, the copy number of MukF (340 ± 100) and MukE (170 ± 50) inside the cell would support the formation of a F2E4 complex (23). The bimodal association of MukF and MukE also explains why both excess and absence of MukF causes defects in chromosome segregation (14), because imbalance of MukF would alter the ratio between the F2E2 and F2E4 complexes.

The structural and functional comparison of MukF (17) with other known kleisins (2, 9, 11, 24, 25) suggests that a dimer of MukF interacts with two MukB subunits. We propose that MukE works as a latch to secure the intrinsically unstable interaction between MukB and MukF (20). Therefore, association of F2E4 with MukB would cross-link MukB either intramolecularly to form rings (Fig. 4) or intermolecularly to form filaments or rosette-like structures (19). Alternatively, binding of F2E2 to MukB or the loss of a MukE dimer from the B2F2E4 complex would allow for a switch from a locked to a functional remodeling complex (Fig. 4). Because B2F2E2 has only one of the MukB-MukF monomers latched by MukE, the complex would be able to open and close at the other arm, facilitating the access of DNA to the inner side of the V-shaped MukB (Fig. 4).

Two different MukBF complexes have been recently identified (23). The first one, with a B2F2E4 stoichiometry, is refractory to DNA binding, reinforcing the idea that MukE latches the complex and thus prevents access to the DNA binding moieties of MukB. The second one, with a proposed B2F1E2 stoichiometry, binds and condenses DNA. These results are
Analysis of the MukFE Complexes

FIGURE 4. The MukF dimer, shown as light and dark blue subunits, interacts with one or two dimers of MukE, shown in light and dark green, to form F2E4 or F2E2 complexes (top). Dimers of MukB, depicted as orange-red dimers, can interact with either one of the MukFE complexes. Interaction of F2E4 (top left) with MukF triggers intra- or intermolecular cross-linking. Intramolecular cross-linking of two MukB heads yields rings with stoichiometry B2F2E4 (bottom left), whereas intermolecular cross-linking generates higher ordered MukB structures as described elsewhere (19). Because MukE latches both arms of MukB to a MukF dimer, the B2F2E4 complex is unable to interact with DNA. F2E2 forms an open complex with MukB where only one of the head domains of MukB is latched to MukF (bottom right), thus facilitating the interaction with DNA (gray line). Alternatively, conformational changes on the inactive B2F2E4 complex can cause the loss of a dimer of MukE, yielding an active B2F2E2 complex.

puzzling because MukF is a monodisperse dimer regardless of the ionic strength and the presence or absence of divalent ions (Fig. 1 and data not shown). Furthermore, the structure of the dimerization region of MukF is a doubly domain-swapped dimer, implying that dimerization is necessary to achieve the correct folding of the protein (17). Rybenkov and co-workers (23) assembled the MukBFE complexes from MukB and co-expressed MukFE, assuming that the latter was a single species of stoichiometry F2E4. Using size exclusion chromatography and sucrose gradient fractionation, they identified a short-lived B2F2E4 complex and a substoichiometric B2F1E2 complex, presumably formed by the loss of half of the F2E4 complex. We have now shown that co-expressed MukFE samples contain both F2E4 and F2E2. If both complexes interact with MukB, then they should form B2F2E4 (546 kDa) and B2F2E2 (494 kDa). Alternatively, one of the two MukFE complexes may not interact with MukB; however, their size exclusion chromatography data do not support this possibility (23). B2F1E2 and B2F2E2 only differ in size by 51 kDa, a difference that is too small to be detected by size exclusion chromatography. Hence, we propose that it is the B2F2E2 complex, rather than the B2F1E2 complex, that remodels DNA.

Considering that the F2E4 and F2E2 complexes are stable, the different stability of the two complexes found by Rybenkov and co-workers must be induced by MukB binding. Kleisins bind SMCs in the vicinity of their ATP binding site (26); therefore, the interaction between MukB and MukF might be affected by subtle conformational changes induced upon ATP binding or hydrolysis. Reasonably, different nucleotide-bound states of MukB may have different affinities by F2E4 or F2E2. Therefore, a number of factors such as the availability of ATP and magnesium or the rate of ATP hydrolysis may determine whether MukB interacts preferentially with F2E2 to form a functional complex. Likewise, these factors may determine whether B2F2E2 gains or B2F2E4 loses a dimer of MukE to modulate the remodeling activity of the complex.

In summary, we have identified two stable MukFE complexes and propose that both can interact with MukB to form a locked B2F2E4 complex and a functional B2F2E2 remodeling complex.

Acknowledgments—We thank J. Ortega, M. Junop, and W. Yang for critical reading of the manuscript.

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