**Escherichia coli** MutS Tetrimerization Domain Structure Reveals That Stable Dimers But Not Tetramers Are Essential for DNA Mismatch Repair *in Vivo*

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The *Escherichia coli* mispair-binding protein MutS forms dimers and tetramers *in vitro*, although the functional form *in vivo* is under debate. Here we demonstrate that the MutS tetramer is extended in solution using small angle x-ray scattering and the crystal structure of the C-terminal 34 amino acids of MutS containing the tetramer-forming domain fused to maltose-binding protein (MBP). Wild-type C-terminal MBP fusions formed tetramers and could bind MutS and MutS-MutL-DNA complexes. In contrast, D835R and R840E mutations predicted to disrupt tetrameric interactions only allowed dimerization of MBP. A chromosomal MutS truncation mutation eliminating the dimerization/tetramerization domain eliminated mismatch repair, whereas the tetramer-disrupting MutS D835R and R840E mutations only modestly affected MutS function. These results demonstrate that dimerization but not tetramerization of the MutS C terminus is essential for mismatch repair.

Errors during DNA replication can result in base-base mispairs and small insertion or deletion mispairs that when left unrepaired give rise to mutations during subsequent rounds of replication. The mismatch repair (MMR)4 pathway normally functions during excision and resynthesis to repair the mismatch (9). Although this repair reaction has been reconstituted *in vitro* (9), the molecular mechanism that coordinates the initial events of MMR (mispair recognition) with downstream events (nicking, excision, and replacement of the error-containing strand) is not well understood. Accordingly, several models have been proposed that attempt to explain the orchestration of these events (10–13).

At high concentrations, MutS proteins from *E. coli*, *Thermus aquaticus*, and *Thermus thermophilus* undergo a dimer to tetramer transition (14–17). The C-terminal region mediates tetramerization and includes the last 53 amino acids in *E. coli* MutS (Fig. 1) (18). C-terminal truncations were used to obtain dimeric crystal structures of MutS and MutS bound to DNA, and hence, little is known about the structure of the C-terminal 53 amino acids of MutS (18, 19). Despite its crystallographic utility, the *E. coli* deletion protein MutSΔ800 has severe biochemical defects in key functions including mispair recognition and MutH stimulation (15). Furthermore, functional complementation of *E. coli* mutS deletion strains by the mutSΔ800 protein for mutation avoidance requires overexpression from plasmids (20). Integration of the mutSΔ800 mutation into the genome demonstrates that this allele results in a substantial MMR defect (21). In contrast, the antirecombination defects in mutSΔ null strains are not complemented by MutSΔ800 protein, even with overexpression (20).

The *in vitro* and *in vivo* defects caused by the C-terminal deletion and the fact that the *E. coli* tetramer disassociation constant is close to the *in vivo* MutS concentration has led to the suggestion that tetramerization is important for MMR (15, 21). However, the MutSΔ800 protein has dimerization defects in addition to tetramerization defects (22, 23). Thus, the ability of MutSΔ800 to complement mutS strains only when overexpressed might be due to stabilization of the dimer (20, 21). Furthermore, the reported association constant for tetramerization ranges over 2 orders of magnitude, from 2.1 × 10−7 M−1 to 2.2 × 10−6 M−1 for *E. coli* (15, 22) and up to 1.3 × 10−5 M−1 for *T. aquaticus* (14). This result is at odds with the idea that MutS concentrations are kept close to the concentration at which tetramers form. Despite this, recent experimental data indicate

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*This work was supported by National Institutes of Grants GM50006 and CA92584. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby indicated "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The atomic coordinates and structure factors (code 2ok2) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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4 The abbreviations used are: MMR, mismatch repair; SAXS, small angle x-ray scattering; MBP, maltose-binding protein.
that a point mutation, D835R, which causes defects in tetramerization of a peptide corresponding to the C terminus of MutS, is genetically identical to mutS801 in an overexpression assay, which has suggested a role for tetramerization in vivo (23).

In the present study we have determined that the MutS tetramer is extended by small angle x-ray scattering (SAXS), and our model explains why MutS does not assemble into complexes larger than tetramers. To gain insight into MutS tetramerization, we fused the C-terminal 53 residues of MutS800–853 to the C terminus of E. coli MutS onto maltose-binding protein (MBP) and showed that the fusions cause monomeric MBP to tetramerize. The crystal structure of the tetramerization domain revealed an extensively associated dimer interface, which made weaker interactions to form tetramers. The MBP fusions bound MutS and MutS-MutL-DNA complexes, and binding required tetramerization motifs on both MBP and MutS. Deletions of the tetramerization motif predicted to affect both dimerization and tetramerization caused substantial MMR defects when the deletion mutations were integrated into the chromosome. In contrast, MutS tetramer-disrupting point mutations were mostly functional when present at the chromosomal mutS locus. Together, our data resolve the controversies surrounding MutS tetramerization by demonstrating that the dimer-stabilizing role of the MutS C terminus is essential for MMR, but that the tetramer-forming role is not.

EXPERIMENTAL PROCEDURES

Plasmid and Strain Construction and General Genetics Methods—A His<sub>6</sub>-MutSΔ800 expression vector was created by replacing the RsrII-BamHI fragment of pTX412 (24) (pET15b–His<sub>6</sub>-MutS; a gift of Malcolm Winkler) with the RsrII-BamHI fragment from MutSΔ800 B5 (gift of Paul Modrich) yielding His<sub>6</sub>-MutSΔ800 in the pET15b expression vector (pRDK1240).

His<sub>6</sub>-MBP was amplified by PCR from plasmid pMYB5 DNA (New England Biolabs) using a forward primer containing a Ncol restriction site followed by a His<sub>6</sub> tag and a reverse primer containing a Ndel restriction site and was ligated into pET15b (Novagen), yielding pRDK1232 (pET15b–His<sub>6</sub>-MBP). The E. coli MutS C-terminal DNA sequence encoding amino acids 801–853 and 820–853 was amplified by PCR with forward primers containing a BamHI restriction site and reverse primers containing a BamHI restriction site and ligated into pRDK1232. This created pRDK1233 and pRDK1234, which fused MutS801–853 and MutS820–853, respectively, to the C terminus of His<sub>6</sub>-MBP. The MBP801-D835R (pRDK1235) and MBP801-R840E (pRDK1236) expression vectors were made as described above, except the mutant mutS was amplified off of the appropriate mutant mutS allele.

The strains for in vivo analysis were constructed by PCR-mediated recombination into an E. coli strain conditionally expressing the bacteriophage lambda Red system (21, 25). Briefly, the bla gene (encoding ampicillin resistance) was amplified using a forward oligonucleotide containing 50 bases of homology to MutS for integration at the desired location in the E. coli MutS locus followed by a stop codon and a reverse oligonucleotide containing an additional 50 bases of homology immediately downstream of the native MutS codon. PCR products were transformed into strain TP798 (25) and resulted in ampicillin-resistant strains with a stop codon followed by the bla gene after residues 11 (RDK4782), 800 (RDK4783), 819 (RDK4784), 834 (RDK4785), and 853 (end of wild-type sequence) (RDK4786) of the chromosomal mutS locus. The mutS D835R (RDK4787) and R840E (RDK4788) point mutations were made by amplifying the bla gene from RDK4786 (MutS wild-type bla) genomic DNA using a forward oligo incorporating the desired point mutation and the same reverse oligonucleotide as above and transforming the cassette back into TP798. All chromosomal integrants were verified by PCR and sequencing.

Mutation Rate Assay—Mutation rates for rifampicin resistance were determined by fluctuation analysis using at least 15 independent cultures for each strain (26–28). Cultures were grown overnight, and dilutions were plated on LB plus 100 μg/ml ampicillin with and without 100 μg/ml rifampicin and allowed to grow overnight at 37 °C. A two-tailed Mann-Whitney test was performed to calculate p values using Graphpad Prism version 4.0b for Macintosh (Graphpad software).

Protein Expression and Purification—His<sub>6</sub>-MutS and His<sub>6</sub>-MutL were expressed and purified essentially as described previously (24), with the addition of a Mono Q column. His<sub>6</sub>-MutSD800 was expressed in BL21(DE3)Tn10:mutS (gift of Paul Modrich). The His-tagged MBP proteins were expressed in BL21(DE3)Tn10:mutS and purified with a nickel column followed by binding to amylose resin and were eluted with 10
mM maltose. Proteins aliquots were frozen in liquid nitrogen and stored at −80 °C.

Gel Filtration Chromatography—From 0.25 to 5 mg of the MBP-MutS fusions were loaded onto a Superdex 75 column (Amersham Biosciences) and run in buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, and 0.5 mM EDTA. Both MutS and MutSΔ800 were loaded onto a Superose 6 column (Amersham Biosciences) at a monomeric concentration of 50 μM and run in buffer containing 20 mM Tris (pH 8.0), 300 mM NaCl, 0.5 mM EDTA, and 4 mM dithiothreitol. Stokes radii were calculated with a linear regression of retention time versus radius using known standards.

Amylose Pull-down Assays—Binding reactions containing 600 pmol of MBP or MBP801 and 600 pmol of MutS or MutSΔ800 were incubated in 50 μl of buffer containing 20 mM Tris (pH 7.5), 4 mM MgCl₂, and 100 mM NaCl on ice for 15 min followed by the addition of 100 μl of amylose resin (New England Biolabs; resuspended in the above buffer) for an additional 15 min. After extensive washes, bound proteins were eluted with 100 μl of buffer containing 20 mM maltose, separated by SDS-PAGE, and stained with Coomassie Brilliant Blue. Experiments analyzing formation of ternary complex with MutL were performed as follows. MBP801 and MutS were incubated and bound to amylose resin, as described above. Samples were then washed with buffer with and without 250 μM ATP as indicated. Next, 300 pmol of MutL and/or 150 pmol of mispair containing DNA (71 bp) in buffer with and without 250 μM ATP were added and incubated for 15 additional minutes on ice as indicated. Samples were washed with buffer with and without 250 μM ATP and eluted and processed above.

SAXS Data Collection and Processing—SAXS data were collected at beamline 12.3.1 at the Advanced Light Source at Lawrence Berkeley National Laboratories. A wavelength of 1.115869 Å was used with a sample to a detector distance of 1.48 meters. The resolution scale was calibrated using a silver behenate calibration standard that had diffraction maxima at 0.107633 Å. Buffer and samples were collected alternately with short exposures bracketing a longer exposure. The short exposures were compared to ensure that no radiation damage occurred, and data from all exposures were merged using PRIMUS. For each sample, data were collected at several different protein concentrations, and the scattering was fit by the indirect Fourier transform method (29) as implemented in GNOM (30). Theoretical scattering from atomic models was compared with experimental data using the program CRYSOL (31).

Crystallization and Structure Determination—MBP820 was crystallized by the hanging drop method against a reservoir of 15% polyethylene glycol 4000, 100 mM sodium citrate (pH 5.6), and 100 mM lithium acetate. X-ray diffraction data were collected alternately with...
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FIGURE 3. The final 34 amino acids of E. coli MutS mediate tetramerization of MBP. A, size-exclusion chromatography of MBP (black), MBP801 (blue), and MBP820 (red) reveals that the C-terminal MutS fusions mediate multimerization of MBP801 and MBP820. B, x-ray scattering of equal monomer concentrations of MBP (black), MBP801 (blue), and MBP820 (red). C, x-ray scattering of monomeric MBP (black points) was compared with calculated scattering of the open (blue line, PDB code 1peb (54)) and closed (red line, PDB code 1fqc (44)) crystal structures of MBP using CRY SOL (31). The experimental scattering (Rg 22.4 Å) fit the closed MBP structure (χ 3.1, Rg 21.97 Å) better than the open structure (χ 10.6, Rg 23.0 Å). Similarly, ab initio reconstruction of the MBP shape generated by GAS BOR (42) fits the experimental scattering (green line) and the closed crystal structure well (average χ = 1.2 ± 0.1, n = 10). D, the ratio of I(0)/I(0)monomer reveals that the complexes formed by MBP801 (blue) and MBP820 (red) are tetrameric. The average ratio is 4.4 ± 0.2 for MBP801 and 3.82 ± 0.08 for MBP820. The I(0) for each protein is linear with respect to concentration (inset). E, pair distribution function calculated for MBP (black), MBP801 (blue), and MBP820 (red).

RESULTS

Full-length MutS Is Tetrameric, whereas MutSΔ800 Is Substantially Monomeric—To establish that preparations of wild-type MutS were tetrameric, both MutS and the MutSΔ800 proteins were analyzed by size-exclusion chromatography (Fig. 2A). Similar to previous observations, the Stokes radius (Rg) of the wild-type tetrameric MutS, 83.3 Å, was larger than would be predicted for a globular MutS tetramer, which has an expected Rg of 59.0 Å and a Mr of 358 kDa (39). By contrast, the MutSΔ800 truncation (monomeric molecular mass of 89.5 kDa) eluted with an Rg of 42.5 Å, similar to a 113-kDa globular protein, which was much smaller than the Rg of 49 Å and Mr of 179 kDa expected for a globular MutS dimer. Dimerization defects in the MutSΔ800 have been previously observed (22, 23). The wild-type protein also discriminated between fully base-paired DNAs and those containing single mispairs and formed MutS-MutL-DNA-ATP complexes as demonstrated in total internal reflectance and surface plasmon resonance experiments, whereas MutSΔ800 had substantial defects even in binding to mispair-containing DNA (data not shown), consistent with previous data (15).

SAXS data collected on wild-type MutS and MutSΔ800 agreed with the size-exclusion chromatography data (Fig. 2B). The radius of gyration (Rg), which is an x-ray analog of Rg, was 38.3 Å for MutSΔ800 and 80.8 Å for MutS. Unlike Rg and Rg, the scattering intensity at zero angle, I(0), is independent of shape and can be used to determine stoichiometry of oligomers. I(0) depends linearly upon protein concentration (Fig. 2C, inset) and depends on the square of the molecular weight of the scattered particle (40). In protein solutions with equal molar concentrations of monomers, multimerization increases the molecular weight and decreases the concentration of scattering particles by the same integral factor. Thus the ratio of I(0) of the multimer to the I(0) of the monomer gives the stoichiometry of the complex. The ratio between wild-type MutS, which is known to form tetramers, and MutSΔ800 was 4.0 ± 0.5 (Fig. 2C), indicating a tetrameric assembly for wild-type MutS and a monomeric assembly for MutSΔ800 under our conditions.

The MutS Tetramer Is Extended—To understand the structure of the MutS tetramer in solution, we calculated the Fourier transforms of the SAXS curves to generate the pair-distribution function, P(R) (Fig. 2D). The P(R) is a histogram of distances between electrons in the scattering particle and can be directly compared with P(R) functions calculated from atomic models.
Potential MutS tetramers constructed with face-to-face or side-to-side contacts of the MutS rings were not consistent with the experimental \( P(R) \) function with a \( D_{\text{max}} \) of \( \sim 250 \) Å. Instead, only elongated tetramers with contacts either at the ATPase domains (head-to-head tetramers) or the DNA clamping domains (tail-to-tail tetramers) were consistent with the experimental \( P(R) \) function. These elongated structures were consistent with the fact that wild-type MutS tetramers had a larger \( R_g \) than would be predicted by the molecular weight of the tetramer (39) (Fig. 2A). Among the potential extended MutS tetramers, the C termini required for tetramerization were brought together only in the head-to-head tetramer and suggested this arrangement over the tail-to-tail or head-to-tail tetramers. This head-to-head tetramer is consistent with the observation that oligomers larger than tetramers were not observed in analytical ultracentrifugation experiments (15, 22).

In contrast to potential multimers formed by head-to-tail, face-to-face, or side-to-side arrangements of MutS rings, a head-to-head tetramer sterically occludes the C-terminal domain mediating the tetramerization and would block the formation of higher order oligomers.

**Addition of the MutS C Terminus Causes MBP to Tetramerize**—The C-terminal 53 amino acid domain could tetramerize MutS by two distinct mechanisms. In the first, the domain might only form a dimer and cause MutS to tetramerize by pairing with a symmetry-related partner from another MutS dimer through an alternative dimer interaction. In the second, the domain itself might tetramerize, thereby bringing together two MutS dimers. To distinguish these possibilities, we fused the last 53 amino acids of *E. coli* MutS (residues 801–853, Fig. 1) to the MBP C terminus to generate MBP801. The purified MBP801 protein eluted earlier in size-exclusion chromatography (\( R_g \) of 52.6 Å) than monomeric MBP (\( R_g \) of 15.7 Å; Fig. 3A). The elution time matched the expected elution time for a MBP tetramer; however, elongated molecules have a larger \( R_g \) than would be predicted by molecular weight alone so this does not constitute strong proof of tetramer formation.

The 53 amino acids in the C-terminal motif can be divided into a non-conserved N-terminal region not predicted to form secondary structure and a C-terminal region predicted to form two amphipathic helices (Fig. 1). A second MBP fusion, MBP820, was generated that contained the region of the C-terminal motif predicted to form secondary structure (residues 820–853). The expressed protein chromatographed almost identically to MBP801 with an \( R_g \) of 46.8 Å (Fig. 3A), which indicated that the last 34 residues are sufficient to increase the \( R_g \) of the MBP fusion.

SAXS data were collected for MBP801, MBP820, and MBP to understand conformation and stoichiometry of the MBP constructs (Fig. 3B). The \( R_g \) was 58.1, 45.7, and 22.1 Å for MBP801, MBP820, and monomeric MBP, respectively. This was consistent with both the \( R_g \) and oligomerization observed by size-exclusion chromatography. MBP exists in an “open” unbound state and a “closed” maltose-bound state (41). Comparison of calculated scattering from crystal structures of known MBP conformations indicated that the MBP monomer was closed (Fig. 3C), similar to previous SAXS results (41). Additionally, *ab in situ* structure generation from the SAXS data using GASBOR (42) fits the known crystal structure of the closed form of MBP.

**Stoichiometric measurement by \( I(0) \) ratios** at equivalent monomer concentrations indicated that MBP801 and MBP820 were tetramers (Fig. 3D), consistent with analytical ultracentrifugation analysis of the isolated MutS peptide comprising residues 801–853 (23). These data show that the C-terminal motif directly mediates tetramerization, which is consistent with the head-to-head extended MutS tetramer solution assembly predicted by the SAXS analysis. Unlike monomeric MBP, the \( P(R) \) function of MBP801 and MBP820 had two peaks, suggesting that the average distances between the centers of the MBP domains in the MBP801 and MBP820 tetramers were 78 and 62 Å, respectively (Fig. 3E). The larger distances observed with MBP801 were consistent with longer linkers distances.

![FIGURE 4. MBP801 can bind MutS and MutS-MutL-DNA complexes.](image)
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MBP801 Can Interact with MutS and MutS-MutL-DNA Complexes—The MBP801 fusion retained the ability of wild-type MBP to bind with high affinity to starch matrices and could be eluted from such matrices with maltose (Fig. 4A). Thus, we sought to determine whether the MBP fusions could specifically bind MutS. MBP801 was bound to a starch matrix, and MutS or MutSΔ800 was added. After washing, the bound proteins were eluted with 10 mM maltose and analyzed by SDS-PAGE. Full-length MutS copurified with MBP801 in the maltose elution but was not eluted from the starch column by maltose in the absence of MBP801. Similarly, MutSΔ800 did not co-purify with MBP801 nor did full-length MutS co-purify with wild-type MBP. Thus, the formation of heterotetramers between MutS and MBP801 was dependent upon the presence of the tetramerization motif.

Because MutS could be immobilized on a starch matrix through interaction with MBP801, we addressed the question of whether or not this interaction would be disrupted by the formation of a ternary complex containing MutS, MutL, and mispaired DNA (Fig. 4B). MutS, MutL, ATP, and mispaired DNA were added to a starch matrix containing prebound MBP801. In the presence of maltose, MutS and MutL co-eluted with MBP801, indicating that ternary complexes can form a complex with MBP801. The binding of MutL to MBP801 required MutS and mispaired DNA and was stimulated by ATP (Fig. 4B), consistent with known requirements for the formation of ternary complexes in vitro (6, 11, 12, 43). Thus, the ternary complex can form with MutS-MBP801 heterotetramer and does not appear to require MutS homotetramerization.

Crystal Structure of MBP820 Reveals the Fold of the Tetramerization Domain—MBP820 crystallized in the monoclinic space group C2 with two molecules in the asymmetric unit, whereas MBP801 did not crystallize even at protein concentrations greater than 100 mg/ml. The 2.0-Å structure of MBP820 was solved by molecular replacement (Table 1), using the closed conformation of MBP as a model (PDB code 1fqc (44)). Electron density clearly showed MBP820-bound maltose, which was likely scavenged during the purification protocol (data not shown).

The crystal structure revealed that the tetramerization domain was generated from a helix-loop-helix structure that is reminiscent of a HEAT repeat (45). Dimerization forms a two-layer structure that packs helices orthogonally. The dimer was symmetric despite the fact that it was generated by non-crystallographic symmetry (Fig. 5A). The dimer interface buried 912 Å² per monomer, which is substantially larger than would be predicted based on the 3.9-kDa size of the domain (46).

Moreover, the interface was extensively hydrophobic and was composed of side chains from residues in both amphipathic helices and the connecting loop. This loop had a defined geometry that is likely controlled by Pro-834, Pro-839, and Leu-837, whose side chain was buried in the central hydrophobic core in a manner similar to the conserved hydrophobe in DNA binding helix-hairpin-helix motifs (47) (Fig. 5B). Surprisingly, the conserved Lys-850 appeared to stabilize the final turn of the first helix in the other monomer of the dimer by neutralizing the helix dipole rather than interacting with negatively charged side chains. Similarly, the conserved Asp-835 formed hydrogen bonds with the Tyr-847 side chain in the dimer and only made salt bridging interactions in the tetramer as described below.

The tetramer interface was generated by a crystallographic 2-fold relating two dimers (Fig. 5C). Unlike the dimerization interface, this surface was relatively small, burying only 233 Å² per monomer. This surface sequestered few hydrophobes and rather remarkably was asymmetric with respect to the individual monomers in the dimer. One-half of the interface was formed by the packing of two loops (Asp-833 to Arg-840) from one monomer of the dimer, and the other half involved packing of the second helix (Arg-840 to Arg-848) in the other monomer. Thus, the individual monomers of the tetramer can be distinguished by being loop-loop-packed monomers or helix-helix-packed monomers. The tetramer buried two side chains of Leu-843 from the helix-helix-packed monomers and two side chains of Pro-839 from the loop-loop-packed monomers; however, the two other copies of each of these residues remain solvent-exposed in the tetramer. Electrostatic interactions also appeared to play an important role at the interface. Two of the four Arg840 residues from loop-loop-packed monomers made favorable salt bridge interactions with the Asp-833 and Asp-835 from the other loop, whereas the other two Arg840 residues from helix-helix-packed monomers stacked against Tyr-847 and made a salt bridge with Glu-844 from the other helix.

Despite the small size of the observed tetramer interface, several lines of evidence suggest that the observed interface causes tetramerization in solution. First, the high dissociation constant measured for MutS tetramerization (15, 22), which is around or above the estimated concentration of MutS in the E. coli cell (48), suggested a small buried surface area. Second, the MBP820 tetramer in the crystal structure was consistent with solution SAXS data, with a calculated Rg for the tetramer of 42.3 Å as compared with an experimental Rg of 45.7 Å (Fig. 5D). For comparison, the calculated Rg of the MBP820 dimer was only 34.9 Å. Differences in measured and calculated scattering profiles of the tetramer suggested that crystal-packing forces...
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compacted MBP820 relative to the average solution conformation. Third, disruption of the electrostatic interactions in the observed tetramer interface, such as by the D835R mutation, previously shown to prevent tetramerization of the C-terminal peptide and MutS (23) as well as by the R840E mutation, disrupted tetramerization without substantially affecting dimerization of the MBP801 fusion (Fig. 5F). Thus, the crystallographically generated tetramerization interface is likely the interface that mediates formation of E. coli MutS tetramers (Fig. 5F).

We noticed that the D835R version of MBP801, but not the R840E version, had an elution profile that was strongly concentration-dependent (data not shown). The elution positions were between those for the MBP monomers and MBP dimers, indicating that the D835R mutant had some dimerization defects. Consistent with this, Asp-835 makes interactions in the dimer, whereas Arg-840 does not. In the context of the full-length MutS protein, however, the D835R mutation did not appear to affect dimerization (23).

Dimerization, but Not Tetramerization, of MutS Is Critical for MMR in Vivo—The structural characterization of the tetramerization C-terminal domains suggests that dimerization should be a stronger interaction than tetramerization. Truncation alleles, however, would be predicted to affect the role of the C-terminal domain in both processes. Thus, to understand the importance of the different functions of the C-terminal domain for MMR in vivo, we introduced mutant alleles of mutS onto the chromosome and tested the mutant strains for increased rates of mutations that give rise to rifampicin resistance, indicative of an MMR defect (21).

Mutation rates of strains bearing the chromosomal mutSΔ800 allele were ~2-fold lower than mutSΔ11 null alleles (Table 2), similar to previously published results (21). Similarly, C-terminal deletions that eliminated successive secondary structural elements, mutSΔ819 and mutSΔ834, were MMR-defective and had mutation rates that were statistically indistinguishable from the mutSΔ800 allele (p = 0.3401 and p = 0.1249, respectively; 2-tailed Mann-Whitney test was used for all pair-wise comparisons). In contrast, a mutS mutant where the β sliding clamp interaction motif was deleted (mutSΔ812–816), in the region directly upstream of the predicted secondary structural elements, did not affect MMR in vivo (49). These results indicated that the secondary structural elements within the C-terminal domain are important for MutS function but do not indicate the relative significance of dimerization and tetramerization in vivo.

To specifically address the role of tetramerization, the point mutants mutS-D835R and mutS-R840E were introduced onto the chromosome. Both of these C-terminal motif mutations cause tetramerization defects without substantially affecting dimerization (23) (Fig. 5E). When integrated onto the chromosome these mutations caused only small MMR defects. These mutation rates were significantly different from the effects of the mutSΔ800 allele (p = 0.0001 for both comparisons). This contradicts a previous study in which the mutS-D835R mutation was found to be indistinguishable from the mutSΔ800 allele (23). However, that study tested plasmid-encoded alleles resulting in increased expression, and it is known that overexpression of mutSΔ800, which encodes a MutS truncation with concentration-dependent dimerization defects, dramatically improves the ability of this allele to complement a mutS null strain (21). Our analysis of chromosomally integrated alleles together with specific mutations that disrupt tetramerization but not dimerization reveals that MutS tetramerization is not essential for MMR function in vivo. Consistent with this, overexpression of the MBP801 fusion to compete for MutS tetramerization in vivo had no effect on the mutation rate (data not shown).

DISCUSSION

In the present study we have shown that the MutS tetramer is generated through an asymmetric tetramerization domain that generates strong dimeric, but weak tetrameric interactions. This domain mediated tetramerization of MBP fusions as well as MBP fusion interactions with MutS and MutS-MutL-DNA complexes. Furthermore, we have shown that the MutS tetramer is extended in solution and when combined with the crystallography of the tetramerization motif reveals why oligomers larger than tetramers are not observed. Moreover, the MutS tetramer derived from SAXS data is remarkably similar to the structures observed by electron microscopy at the bases of DNA loops (10), assuming that these structures correspond to two MutS dimers, with each bound to different parts of the DNA molecule as has been suggested by others (1, 15, 50).

Finally, our genetic data revealed that the tetramerization motif is required for MMR in vivo, presumably through stabilizing MutS dimers, but that formation of MutS tetramers by this domain is not significantly required for MMR.

After the asymmetric recognition of mispairs by MutS (18, 19) and formation of a MutS-MutL-DNA ternary complex (6, 11, 12, 43), the downstream events in MMR are poorly understood. In the methyl-directed mismatch repair system in E. coli, MutS and MutL somehow activate the MutH endonuclease, whereas events in other bacterial and eukaryotic systems are more obscure. At least three competing models for MutS and/or MutS-MutL signaling have been proposed (10–12); they are bidirectional ATP hydrolysis-dependent MutS translocation, ATP hydrolysis-independent MutS sliding and signal transduction, and ATP-dependent authorization of MutS mispair recognition that does not involve dissociation of MutS from the mispair. Tetramer formation by MutS has been suggested to support the translocation model (15) or in models where one dimer remains bound to the mispair, and the other dimer is involved in the strand discrimination signal (50, 51).

| Strain   | Relevant genotype | Mutation rate (Rif R) |
|----------|-------------------|----------------------|
| RDK4786  | Wild type         | 1.0 (0.7–1.4) × 10⁻⁴ (1) |
| RDK4782  | mutSΔ11           | 4.5 (3.3–7.5) × 10⁻⁷ (44) |
| RDK4783  | mutSΔ800          | 1.9 (1.3–2.7) × 10⁻⁷ (19) |
| RDK4784  | mutSΔ819          | 2.2 (1.4–5.6) × 10⁻⁷ (22) |
| RDK4785  | mutSΔ834          | 3.0 (1.9–4.2) × 10⁻⁷ (29) |
| RDK4787  | mutS-D835R        | 4.5 (3.5–7.2) × 10⁻⁷ (4) |
| RDK4788  | mutS-R840E        | 5.2 (2.7–5.7) × 10⁻⁵ (5) |

* The numbers in brackets represent low and high values, respectively, for the 95% confidence interval for each rate. The number in parentheses indicates rate relative to wild-type rate.
Our data indicating that the dimerization/tetramerization domain, but not tetramerization per se, is required for MMR suggest that aspects of these models may need revision. For example, MutS may drive reactions in vitro, such as formation of large tetramer-restrained DNA loops during ATP-dependent translocation (10), which may not be required for most MMR events in vivo. Our conclusions are at odds with the interpretation of others for the relevance of tetramerization to MMR (15, 23); however, our conclusions are fully consistent with their data given that the MutSΔ800 protein has dimerization as well as tetramerization defects (22, 23) and that mutSΔ800 can complement MMR defects in mutSΔ null strains but only when overexpressed (20).

In addition, previous studies have shown that whereas the MutSΔ800 protein has a lower affinity for mispaired DNA, it retains specificity for mispaired DNA versus homoduplex DNA and is only weakly functional in in vitro MMR reactions (15). In light of our data and other previously published data, this may not be surprising. A significant amount of MutSΔ800 protein might be expected to be monomeric (monomer to dimer $K_d = 13 \mu M$ (22)) due to lack of stabilization by the dimerization domain especially at the submicromolar concentrations of protein that were used in these assays. This is consistent with in vivo results from MMR complementation assays, where normal levels of expression of the mutSΔ800 allele from a plasmid largely complements MMR defects of mutSΔ null strains but only when present on the chromosome, whereas they behave identically in complementation assays when both alleles are expressed at higher levels from plasmids.

We have observed that although the portion of the C-terminal domain responsible for stabilizing the dimer is conserved in MutS proteins from the majority of bacteria, the charged amino acids contributing to the salt bridges that are essential for tetramerization of the E. coli MutS protein are conserved only in a subset of the proteobacteria. Consistent with this, MutS from T. thermophilus, which lacks predicted salt-bridge-forming residues (Fig. 1), tetramerizes with a dissociation constant that is 2 orders of magnitude higher than the E. coli protein (14, 15). Moreover, we are unaware of any reports describing the tetramerization of eukaryotic Msh2-Msh6 and Msh2-Msh3 MutS homologue complexes, which lack this C-terminal domain that mediates both dimerization and tetramerization, consistent with tetramerization being dispensable for conserved MMR functions. We do note, however, that MutS function in MMR and homologous recombination events appear to be differentially affected by the mutSΔ800 allele (21), which is substantially different from engineered mutations in other regions of the MutHLS proteins, where no difference in the two processes were noted (52).

Acknowledgments—We thank Paul Modrich, Malcolm Winkler, and Anthony Poteete for generous gifts of plasmids and bacterial strains, Jeff Perry and Stanford Synchrotron Radiation Laboratory beamline staff for assistance in crystallographic data collection, and Greg Hura for assistance in SAXS data collection. We also thank Scarlet Shell for helpful discussions and comments on this manuscript.

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