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Contribution of Msh2 and Msh6 subunits to the asymmetric ATPase and DNA mismatch binding activities of *Saccharomyces cerevisiae* Msh2–Msh6 mismatch repair protein

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Abstract

Previous analyses of both *Thermus aquaticus* MutS homodimer and *Saccharomyces cerevisiae* Msh2–Msh6 heterodimer have revealed that the subunits in these protein complexes bind and hydrolyze ATP asymmetrically, emulating their asymmetric DNA binding properties. In the MutS homodimer, one subunit (S_1) binds ATP with high affinity and hydrolyzes it rapidly, while the other subunit (S_2) binds ATP with lower affinity and hydrolyzes it at an apparently slower rate. Interaction of MutS with mismatched DNA results in suppression of ATP hydrolysis at S_1—but which of these subunits, S_1 or S_2, makes specific contact with the mismatch (e.g., base stacking by a conserved phenylalanine residue) remains unknown. In order to answer this question and to clarify the links between the DNA binding and ATPase activities of each subunit in the dimer, we made mutations in the ATPase sites of Msh2 and Msh6 and assessed their impact on the activity of the Msh2–Msh6 heterodimer (in Msh2–Msh6, only Msh6 makes base specific contact with the mismatch). The key findings are: (a) Msh6 hydrolyzes ATP rapidly, and thus resembles the S_1 subunit of the MutS homodimer, (b) Msh2 hydrolyzes ATP at a slower rate, and thus resembles the S_2 subunit of MutS, (c) though itself an apparently weak ATPase, Msh2 has a strong influence on the ATPase activity of Msh6, (d) Msh6 binding to mismatched DNA results in suppression of rapid ATP hydrolysis, revealing a "*cis*" linkage between its mismatch recognition and ATPase activities, (e) the resultant Msh2–Msh6 complex, with both subunits in the ATP-bound state, exhibits altered interactions with the mismatch.

Keywords: Mismatch repair; MutS; Msh2–Msh6; ATPase kinetics

1. Introduction

DNA mismatch repair is an important, widely conserved mechanism for maintaining the integrity of genetic information over generations. This repair mechanism corrects base substitution and insertion/deletion mismatches that occur due to errors in DNA replication and recombination, as well as DNA lesions resulting from a variety of internal and external stresses. Repair initiates with MutS protein in prokaryotes, or MutS homologues in eukaryotes (e.g., Msh2–Msh6, Msh2–Msh3), binding the site of the mismatch in duplex DNA. This recognition event triggers excision of the error-containing DNA strand past the site of the mismatch, which is followed by DNA resynthesis and ligation to complete the repair process [1–3].

In addition to their mismatch recognition activity, MutS/Msh proteins also possess an ATPase activity that is essential for DNA repair [4–7]. ATP binding and hydrolysis appear to modulate the interactions between MutS/Msh and DNA as well as other proteins in the repair pathway; thus, understanding how MutS/Msh proteins utilize ATP is necessary for understanding how they function in DNA mismatch repair. Several model mechanisms have been proposed for MutS/Msh action upon mismatch recognition: (a) MutS/Msh proteins translocate on DNA, fuelled by ATP binding and hydrolysis, possibly to interact with other proteins on DNA and coordinate mismatch recognition with downstream events such as initiation of strand excision and DNA resynthesis [8–10]; (b) upon binding ATP MutS/Msh proteins form sliding clamps that diffuse freely on DNA, again, to contact downstream repair proteins and direct repair [11,12]; (c) MutS/Msh proteins utilize ATP binding and hydrolysis to modulate their interaction with DNA, while remaining at the mismatch to direct repair [13–17]. At present, experimental data

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are available in support of each of these very different model mechanisms, therefore the investigation into MutS/Msh DNA binding and ATPase activities continues.

Recent studies from several research groups, including our own, have revealed clear differences between the ATP binding and hydrolysis activities of the two subunits in the MutS/Msh dimer [18–21]. For instance, in *Thermus aquaticus* MutS, one subunit binds nucleotide (ATPγS) with about 10-fold higher affinity than the other subunit (K_D = 3 μM versus 27 μM). Also, the high-affinity subunit hydrolyzes ATP at >30-fold faster rate than the low-affinity subunit (10 s^{-1} versus 0.2–0.3 s^{-1} at 40 °C) [18]. These differences are striking especially since MutS is a homodimer; however, they are in accord with known differences in the DNA binding activities of the two MutS subunits (e.g., conserved phenylalanine and glutamate residues from only one subunit undergo base stacking and hydrogen bonding interactions with the mismatch, respectively) [22,23]. In fact it appears that the asymmetry in the ATPase sites is linked to asymmetry in the interactions of the two subunits with DNA [24,25]. Consistent with this hypothesis, binding of mismatched DNA to MutS specifically suppresses the catalytic activity of the high-affinity subunit, such that the rate of ATP hydrolysis is reduced from 10 to 0.3 s^{-1} [18]. The exact nature and function of asymmetry in the MutS dimer is not clear as yet, but the characteristic appears to be important for DNA mismatch repair as it is conserved among a variety of organisms. For instance, subunits of the *E. coli* MutS homodimer also exhibit differences in their interactions with nucleotides and with mismatched DNA [20,21]. The eukaryotic Msh2–Msh6 heterodimer is no different, as the subunits bind nucleotides with differing affinities [19,26], only one subunit catalyzes rapid ATP hydrolysis (*Saccharomyces cerevisiae* Msh2–Msh6: 2–3 s^{-1} at 20 °C) [19], and only Msh6 contains the conserved phenylalanine residue that can make specific contact with the mismatch in DNA [27,28]. As in the case of *T. aquaticus* MutS, mismatched DNA binding strongly suppresses the activity of the rapid ATP-hydrolyzing subunit in *S. cerevisiae* Msh2–Msh6 (the rate constant decreases from 2–3 to 0.1–0.2 s^{-1} at 20 °C) [19]. It is not known yet which of the two subunits, Msh2 or Msh6, catalyzes rapid ATP hydrolysis and, therefore, which one's activity is altered so dramatically following mismatch recognition by Msh2–Msh6.

Previous studies have probed the ATPase activity of both Msh2 and Msh6 subunits by mutating conserved residues in their active sites for ATP binding and hydrolysis. The results confirmed that the ATPase activities of both Msh2 and Msh6 are required for DNA mismatch repair, and also highlighted differences between the two subunits [6,29,30]. Thus, the effects of mutating the Walker A motif (GxxxxGKS), which coordinates the phosphate groups of ATP, and Walker B motif (DExx), which coordinates the Mg^{2+} ion essential for catalysis, differed depending on whether Msh2 or Msh6 was changed. Substitution of the conserved Walker A glycine with aspartate, or Walker B glutamate with alanine, in Msh6 reduced the ATPase activity of *S. cerevisiae* Msh2–Msh6 to a greater extent than did identical mutations in Msh2 [6,29]. Similar results were obtained with a Walker A lysine to arginine mutation in human Msh6 versus Msh2 [30]. All these studies indicated that the Msh6 subunit contributes "more" than Msh2 to the overall ATPase activity of Msh2–Msh6. However, since the ATPase experiments were all performed in the steady state regime, i.e., they measured the rate-limiting step following ATP hydrolysis, the exact contribution and role of each subunit's ATP binding and hydrolysis activity in the Msh2–Msh6 ATPase mechanism, including the identity of the subunit that catalyzes rapid ATP hydrolysis, remain unknown.

Here we report pre-steady state analysis of the ATPase activities of wild type and mixed wild type-Walker A/B mutant heterodimers of Msh2–Msh6, carried out in order to answer questions such as: (a) which subunit catalyzes rapid ATP hydrolysis and which one has the apparently slower activity? (b) does ATP binding and/or ATP hydrolysis by Msh2 influence ATP binding and/or ATP hydrolysis by Msh6, and vice versa? (c) how is Msh2–Msh6 ATPase activity linked to mismatch recognition, given that only Msh6 makes base specific contacts with the mismatch? The answers reveal complex coordination between Msh2 and Msh6 activities that is likely important for Msh2–Msh6 function in DNA mismatch repair.

2. Materials and methods

2.1. DNA and nucleotides

Synthetic oligodeoxyribonucleotides (37-nucleotide template and G:T complement) were purchased from Integrated DNA Technologies, purified by denaturing polyacrylamide gel electrophoresis, and annealed to prepare a G:T mismatch-containing duplex, as described [19]. pET11a vector was purchased from Novagen and pLANT 2b/RIL was a gift from Michael O'Donnell (The Rockefeller University) [31]. Radioactive nucleotides [α-^{32}P]-ATP, [γ-^{32}P]-ATP, and [^{35}S]-ATPγS were purchased from Perkin-Elmer Life Sciences, and non-radioactive nucleotides were purchased from Sigma Chemicals Co. DNA was labeled with ^{32}P as described previously [19].

2.2. Proteins

Point mutations were introduced in *MSH2* and *MSH6* genes (contained in pET11a or pLANT2b/RIL vectors) using overlap-extension PCR or the QuikChange site-directed mutagenesis kit (Stratagene) and verified by sequencing the entire gene. Mixed wild type–mutant Msh2–Msh6 dimers were co-expressed and purified from *E. coli* as described previously for wild type Msh2–Msh6 [19]. Restriction enzymes and T4 polynucleotide kinase were purchased from New England BioLabs.

2.3. Nucleotide and DNA binding assays

ATPγS binding to Msh2–Msh6 was measured by nitrocellulose membrane binding assays as described previously [19]. Briefly, the membranes (Schleicher and Schuell) were washed with 0.5N NaOH and equilibrated in binding buffer (50 mM Tris–HCl, pH 8.0, 5 mM $MgCl_2$, 5% glycerol). Msh2–Msh6 (2 μM) was incubated with 0–200 μM ATPγS + 0.3 μCi [^{35}S]-ATPγS for 15 min at 25 °C (15 μl reactions in binding buffer; 110 mM final NaCl concentration). Ten microliters of each reaction was filtered through the membrane and 1 μl was spotted onto a separate membrane to measure total nucleotide in the reaction. The molar amount of nucleotide bound to protein was determined and plotted versus nucleotide concentration. The binding isotherms were fit to equations describing 1:1 or 2:1 binding of ligands to macromolecules [18].

Dissociation of ATP from Msh2–Msh6 was measured by incubating Msh2–Msh6 (2 μM) with 200 μM ATP + 0.3 μCi [α-^{32}P]-ATP in the binding buffer for 30 s at 25 °C (110 mM final NaCl concentration), followed by addition of 5 mM Mg^{2+}-ATP chase and filtration of 10 μl aliquots through the membrane at 30 s intervals (up to 5 min). The molar amount of nucleotide bound to the pro-