Investigating the functional coupling between Rad50 ATP active sites

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Investigating the functional coupling between Rad50 ATP active sites

by

Katherine Mary Johnson

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Biochemistry

Program of Study Committee:
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The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this thesis. The Graduate College will ensure this thesis is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University
Ames, Iowa
2018

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ABSTRACT

The Mre11/Rad50 complex plays an essential role in the repair of DNA double-strand breaks by initiating the processing of the DNA ends. The MR complex is made up of two subunits of the Mre11 nuclease and two subunits of the Rad50 ATPase. The precise role of Rad50’s ATPase activity is unclear. Some reports suggest that the ATP acts as an allosteric switch that turns on the activity of Mre11, while others suggest ATP is used to power translocation or unwinding of DNA. To test this hypothesis in Rad50, we developed a method to create heterodimeric Rad50 so that ATP sites can be individually controlled. Using two active site mutations (E505Q and D512A), a heterodimeric Rad50 containing a single functioning ATP active site was generated and purified. Characterization of the single ATP site heterodimer indicates that in the absence of Mre11 and DNA, the two active sites act in an independent fashion. Upon complex formation with Mre11 and DNA, the active sites now display strong coupling, such that DNA no longer acts as an allosteric activator of Rad50 ATP hydrolysis. Additionally, it was found that the MR complex with a single ATP site is no longer able to perform multiple nucleotide excisions during a single binding event. Overall, these results support the hypothesis that the MR complex acts as a motor protein that requires coordination between ATP sites for processive nuclease activity.
CHAPTER I: INTRODUCTION

Double-strand Break Repair-

DNA is constantly subjected to internal and external agents that cause DNA damage [1]. Examples of internal agents are reactive oxygen species and tightly bound proteins that cause stalling and breakdown of the replication fork. External DNA damaging agents include ionizing radiation, UV radiation, and genotoxic chemicals [2]. One of the most harmful types of DNA damage is a double-strand break (DSB), where breakage of the phosphate backbone in each DNA strand allows the ends to physically separate from each other [3]. DSBs are very harmful to the cell because the broken ends can be incorrectly repaired, which may lead to genomic instability, changes in cellular identity, and possibly cell death [4]. Moreover, the cellular replication and transcriptional machinery rely on an uninterrupted DNA backbone for their translocation along their DNA templates and therefore DSBs prevent these fundamental cellular processes.

When DSBs are encountered during DNA replication, the replication fork will collapse and therefore it is essential to effectively repair these breaks [5]. Repair of the DSBs can be achieved through either non-homologous end-joining (NHEJ), which is error-prone but typically happens in non-replicating cells, or homologous recombination (HR), which is error-free but requires a homologous DNA template and is the dominant pathway in replicating cells [6]. NHEJ is the simplest mechanism to repair DSBs. The pathway involves proteins Ku70 and Ku80 that form a heterodimer and bind to the DNA ends, which protects the ends from degradation [7]. When the DSB ends are “clean” (i.e., contain an unmodified 3’ hydroxyl and 5’ phosphate group), they do not require any additional processing before ligation of the broken
ends. Therefore, NHEJ is best for this type of break; however, if the break has more extensive damage that can’t be directly ligated, the repair of the break will require the use of a sub-pathway of NHEJ called microhomology mediated end-joining (MMEJ) or HR [8].

MMEJ aligns the broken DNA ends before ligation using microhomologies and is active during the entire cell cycle [7]. The five steps of MMEJ mechanism are: 1) resection of the DSB ends in order to expose ssDNA overhangs, which is carried out by the Mre11/Rad50 (MR) complex; 2) annealing of the two microhomologous with each other; 3) removal of heterologous flaps; 4) fill-in DNA synthesis; and 5) end ligation [9], [10].

In addition to the role of HR in repairing DSBs, it is also essential in meiosis for maintaining replication forks, chromosome segregation, and preserving telomeres [11]. Repair DSBs via HR occurs during the S phase and G2 phase after DNA replication and the sister chromatid acts as the repair template [7]. Homologous recombination uses an undamaged homologous DNA template for DSB repair [12]. HR is a multistep pathway that involves many proteins, including the MR complex. The first step is DSB resection of the 5' strand to produce a 3' ssDNA overhang [13]. This requires the dual action of the MR complex and Exonuclease I. The initiation of processing between with an endonuclease cut on the 5’ strand approximately 20 nucleotides from the DNA end. Then the MR complex uses its 3’ to 5’ dsDNA exonuclease activity to degrade the 5’ strand in the direction of the DNA end [14]. Exonuclease I is then loaded onto the short 3’ ssDNA overhang and proceeds to degrade the opposite DNA strand in the 5’ to 3’ direction. Rad51 and Rad52 then forms a filament on the 3' ssDNA overhang, which begins strand invasion of the homologous DNA template [15]. DNA synthesis begins using a strand of the homologous chromosome as the template. The repair pathway is completed by
dissociation and annealing of the synthesized DNA strands or processing of the Holliday junction with a DNA nuclease and ligase [16].

Interestingly, bacteriophage T4, along with other related phages, requires HR because the intermediates are essential to initiating DNA replication forks, especially when unfavorable growth conditions are present [17]. As expected, the initial steps of HR in bacteriophage T4 relies on homologs of eukaryotic Rad50 and Mre11 proteins, called gp46 and gp47, respectively. The life cycle of bacteriophage T4 is an important factor of DNA replication and double-strand break repair [18]. The T4 recombination-dependent replication (RDR) system is able to restart inactive replication forks. The RDR system plays a large role of DSB repair in T4-infected cells and the repaired products showed that they had been replicated using HR intermediates [19].

*The Mre11 and Rad50 Proteins*

The MR complex is required for both the HR and MMEJ pathways and is responsible for detecting DSBs and, in eukaryotes, activating the ATM checkpoint [20]. The Mre11 subunit is a dimer requires two Mn$^{2+}$ cations for its nuclease activities. These nuclease activities include hairpin endonuclease, single-stranded DNA (ssDNA) endonuclease, and 3’-5’ double-stranded DNA (dsDNA) exonuclease activity [21], [22]. Mre11 is made up of three domains, the core, the capping, and the Rad50 binding domain (RBD). The core domain contains the nuclease active site and makes a small number of contacts with the Rad50 dimer. The capping domain sits on top of the nuclease activity site and has been shown to bind to DNA. The capping domain is able to rotate the DNA helices so that they are positioned properly and it may be involved in guiding ssDNA to active site [23]. The Rad50 binding domain forms a helix-turn-helix structure and binds tightly the base of the Rad50 coiled-coil domain. This makes up the majority of the
Rad50-Mre11 interactions, as the RBD is necessary and sufficient for complex formation. Spanning the RBD and cap domains is a negatively charged flexible linker, which acts in an autoregulatory fashion by competing with the DNA binding site on Mre11 [24].

Rad50 is a member of the structural maintenance chromosome (SMC) family, which are in turn members of the ATP Binding Cassette (ABC) superfamily of ATPases [25]. All ABC proteins are dimeric and are defined by six highly conserved motifs, all of which come together to form the ATP active sites at the dimer interface [26]. The ATP active sites of ABC proteins are shared, meaning that the amino acids of the Walker A, Walker B, Q-loop, and H-loop motifs from one subunit form a functional ATP active site with the Signature-loop and D-loop from the other subunit (Figure 4). The function of ABC proteins vary widely, but most act as membrane transporters or as DNA repair proteins [27]. In all cases, the highly conserved ABC dimer is appended to other structures or subunits to carry-out their specific activities. In the case of Rad50, the ABC domain is split by an antiparallel intramolecular coiled-coil. At the apex of the coiled-coil lies the zinc-hook motif, which is made up of two absolutely conserved cysteines residues. The Tainer lab has shown that two zinc-hooks come together to form a Zn2+ binding site that resembles the tetrathiolate linkage found in zinc-finger proteins. The affinity of the zinc-hooks for Zn2+ is extraordinarily high, with a Kd in the pico to femptomolar range [28]. The coiled-coil/zinc-hook is thought to be involved in the tethering of DNA strands to each other [14]. Two primary models exist for how Rad50 is involved in DNA tethering. The first model is based on the SMC proteins, where the coiled-coil domain traps DNA between the coiled-coils of the Rad50 dimer. In this scenario, Rad50 would be tethering the broken strand to the homologous DNA repair template. In the second model, two Rad50 dimers hold the two ends of the DSB together in order to facilitate their coordinated repair. Imaging studies, such as EM and
AFM, suggest that Rad50-mediated DNA coupling is dynamic and may involve both of the above models [29].

The MR Complex-

As far as we know, Rad50 and Mre11 do not have any cellular functions on their own and to date no organism containing just one of these genes has ever been identified. It is expected that these proteins form a highly coupled complex with each subunit relying on the other subunits for full activity. It is known that the primary conduit for communication between Rad50 and Mre11 is the coiled-coil domain[14], [28], [30], [31]. The coiled-coil is located at the base of the Rad50 ABC domain and bound by the C-terminal RBD of Mre11. The ATP hydrolysis activity of Rad50 alters the conformational of the coiled-coil and these structures changes are then thought to propagate to the Mre11 subunits. When ATP binding occurs between the two Rad50 subunits it induces rotation of the helix-loop-helix and coiled coils, which creates the clamp conformation when there is increased DNA binding activity [32].

In isolation, Mre11 is a very poor nuclease, with low affinity for DNA, and can only degrade DNA in a non-processive fashion. Likewise, Rad50 by itself is a slow ATPase, with a turnover number of approximately 0.15 sec\(^{-1}\). When Rad50 and Mre11 form a complex, the ATPase activity of Rad50 increase 20-fold, the nuclease activity greatly increases, and the complex becomes a processive exonuclease [33]. It is unclear as to why the Rad50 ATPase activity increases, but it is known that it is not directly related to Mre11 nuclease activity since the presence of Mn\(^{2+}\) is not required for activation [24]. In the absence of exonuclease reaction, dsDNA can be considered V-type allosteric activator because the K\(_m\)-ATP is similar with and
without DNA [33]. A V-type system is an allosteric response to allosteric binding of an enzyme that results in altered catalysis of the enzyme [34].

_Coupling of ATP Active Sites_

A great deal of attention has been paid to potential coupling of the two ATP active sites of the ABC domain. A confusing picture has emerged, where some ABC proteins appear to exhibit strong asymmetry in ATP hydrolysis, even to the point of showing half-site reactivity (i.e., only one ATP site is functional). Based on this work, and the fact that it was initially thought that Rad50 was a very poor ATPase, Tainer and colleagues suggested that ATP binding may act as a switch that turns on the nuclease activity of Mre11. In this model, the MR complex bound to ATP is an active exonuclease and processively removes nucleotides until ATP is hydrolyzed and the complex dissociates from DNA [35]. In this case, Rad50 would not be considered a motor protein, as the energy of ATP hydrolysis is not used to drive movement along the DNA, it merely regulates DNA affinity. It should be noted that many processive exonucleases, such as ExoI and λ exonuclease, do not require ATP hydrolysis to move along their DNA substrates.

Later, an alternative model was presented by Paull and colleagues that is somewhat analogous to the way the RecBCD complex functions. In RecBCD, ATP hydrolysis serves to drive two helicase subunits (RecB and RecD) and the nuclease domain of RecB “goes along for the ride” [36]. This model was supported by the demonstration that Rad50 has a weak dsDNA unwinding activity [37]. DNA unwinding activity would likely require strong coordination between both active sites, as multiple subunit helicases hydrolyze ATP in a sequential fashion.
DNA unwinding activity has not been detected for the bacteriophage T4 Rad50, with or without Mre11. Instead, the Nelson lab has suggested that ATP hydrolysis powers translocation of the MR complex along the ssDNA product and allosterically regulates the activity of Mre11 [33], [38], [39]. Similar to dsDNA unwinding, translocation of Rad50 along ssDNA should require coupling between ATP sites.

To investigate the possibility of coupling between Rad50 subunits and distinguish between an ATP-dependent switch mechanism and ATP-driven ssDNA translocation, we set out to generate a Rad50 heterodimer with only a single functioning ATP site. If the ATP sites are independent from each other, we would expect that the single functioning ATP site would operate at a normal rate (i.e, the complex should be 50% active), whereas if coupling exists the loss of one ATP site should reduce the ATP hydrolysis rate of the other active site. We found that in the absence of Mre11 and DNA, the two active sites act in an independent fashion even with the single ATP site heterodimer. However, it was found that upon complex formation with DNA and Mre11, the active sites show strong coupling and DNA no longer allosterically activates ATP hydrolysis. We also found that during a single binding event, the single active site MR complex is unable to execute nucleotide excisions. These results support the hypothesis that coordination between ATP active sites are required for the MR complex to act as a motor protein for processive nuclease activity.
References


Figure 1. **Regulation of repair pathway choice.** The three modes of DSB repair are outlined above. Modified DNA ends cannot be repaired by nonhomologous end joining (NHEJ), but free ends are able to be processed by any pathway. [7]
Figure 2. **Pathways of DNA double-strand break repair by homologous recombination.** DNA Double-strand breaks can be repaired by distinctive homologous recombination pathways. 

(a) After DSB formation, resection of the DNA ends produce a 3’ single-strand DNA overhang. The 3’ overhang becomes the substrate for strand invasion of a homologous chromosome. Strand invasion occurs to form a nascent D-loop and DNA synthesis begins. 

(b) The D-loop is unwound and the new dsDNA strand anneals with the complementary ssDNA from the other DSB end. 

(c) Alternatively, the Holliday junction is processed with a DNA nuclease and ligase resulting in either noncrossover or crossover products. [11]
Figure 3. **Model for MMEJ and alternative end-joining repair.** During the initial stages of MMEJ, Ku70–Ku80 (green) and Rad51 (red) are removed, which enables 5′–3′ resection by the MRX complex, Sae2 and ExoI reveals microhomologous sequences that anneal to each other. (i) When the annealing is stable, the repair is carried out via flap trimming, fill-in DNA synthesis and ligation, which results in a deletion. (ii) Alternatively, one or more translesion polymerases extend the annealed sequences by using error-prone synthesis. Initial microhomologies are dissociated and realignment of the other microhomologous sequences is followed by flap trimming, fill-in DNA synthesis and ligation completes repair, which results in a deletion plus insertion event. [6]
Figure 4. **ATP active sites.** ABC proteins have shared ATP active sites, which means that the amino acids of the Walker A, Walker B, Q-loop, and H-loop motifs of one subunit form a functional ATP active site with the Signature-loop and D-loop from the other subunit.
Figure 5. **Mre11/Rad50 complex.** Mre11 is represented by the dark blue and yellow subunits and Rad50 is represented by the green and light blue subunits. The structure of the coiled coil is unknown and it is represented by the black lines. The Zn$^{2+}$ (orange sphere) hook domain is located at the top of the structure.
CHAPTER II: INVESTIGATING THE FUNCTIONAL COUPLING BETWEEN RAD50 ATP ACTIVE SITES

Abstract

The Mre11/Rad50 complex plays an important role in the repair of DNA double-strand breaks by initiating the processing of the DNA ends. The MR complex composed of two Mre11 nuclease subunits and two Rad50 ATPase subunits. The precise role of Rad50’s ATPase activity is unclear. Some reports suggest that the ATP acts as an allosteric switch that turns on the activity of Mre11, while others suggest ATP is used to power translocation or unwinding of DNA. Rad50 is an ABC protein, several of which exhibit "half-of-sites" reactivity, where only a single functioning ATP subunit is necessary for full activation. To test this hypothesis in Rad50, we developed a method to create heterodimeric Rad50 so that the two ATP sites can be individually controlled. Heterodimeric Rad50 is obtained by expressing two copies of Rad50 gene within *E. coli* BL21(DE3) cells. One gene produces Rad50 protein with a hexahistidine tag and the other produces a protein with a hexaglutamate tag. The co-expression of these genes produces two homodimeric Rad50 proteins (His₆/His₆ and Glu₆/Glu₆) and one heterodimer protein (His₆/Glu₆). The heterodimer is separated from the homodimers using nickel-agarose and SP-sepharose columns. Native gel electrophoresis suggests that the isolation of the heterodimer was successful. Using two active site mutations (E505Q and D512A), a heterodimeric Rad50 containing a single functioning ATP active site was generated and purified. Characterization of this heterodimer indicates that both Rad50 ATP sites are functional, and in the absence of Mre11 and DNA, the two active sites act in an independent fashion. Interestingly, when Mre11 and DNA are included in the reaction, the active sites now show strong coupling, such that DNA no longer increases Rad50’s ATPase activity. Additionally, it was found that the
MR complex with a single ATP site is no longer able to perform multiple nucleotide excisions during a single binding event. Overall, these results support the hypothesis that the MR complex acts as a motor protein that requires coordination between ATP sites for processive nuclease activity.

**Introduction**

DNA is constantly undergoing damage by a wide variety of internal and external DNA damaging agents. It is important that the DNA is repaired properly, otherwise it can result in further damage leading to diseases and cancer [1]. There are many different pathways involved in the DNA damage repair, including homologous recombination (HR), mismatch repair, nucleotide excision repair, DNA strand crosslink repair, and non-homologous end-joining (NHEJ) [2]. The most severe form of DNA damage is double strand breaks (DSBs), which occur approximately 50 times per day in each cell of the human body. Environmental mutagens, ionizing radiation, metabolic intermediates, and UV radiation are all factors that cause DSBs in DNA [3]. If the DSBs are not repaired it can lead to replicative cell death and apoptosis, whereas if the strands are repaired incorrectly it can result in genomic instability and carcinogenesis [4].

HR and microhomology-mediate end-joining (MMEJ, a specialized type of NHEJ) are two pathways involved in DSB repair. [5] There are many proteins involved in each pathway; however, the Mre11/Rad50 (MR) complex is the only protein that plays important roles in both pathways [6]. The MR complex is involved in initiating the processing of the DNA ends for both HR and MMEJ. The MR complex is made up of two Mre11 nuclease subunits and two Rad50 ATPase subunits.
Consistent with its important role in DNA repair, mutations in Rad50 are associated with childhood leukemia, head and neck cancers, and ovarian cancer. Rad50 is a member of the ATP Binding Cassette (ABC) superfamily, which are defined by six highly conserved motifs that make up the ATP active site [7]. The members of the ABC superfamily are very diverse, but the majority of them are membrane transporters or DNA repair proteins [8]. ABC proteins are obligate dimers having shared ATP active sites. The globular domains of all the ABC proteins have a similar dimeric structure but each of them are attached to additional domains and/or subunits that are specific for their function [9]. In addition to binding Mre11, Rad50 contains the unique coiled-coil domain that is thought to tether DNA through a completely conserved “zinc-hook” motif made up of a cysteine, any two amino acids, then another cysteine (CXXC) [8]. The zinc-hook acts as a second dimerization domain, such that two cysteines from each subunit come together to bind a Zn$^{2+}$ cation. The coiled-coils are thought to be involved in the tethering of DNA to each other, likely in order to keep the two broken ends of a DSB together [10].

Mre11 is a Mn$^{2+}$-dependent dsDNA exonuclease and a ssDNA endonuclease [11][12]. In humans, mutations of the Mre11 causes hypersensitivity to ionizing radiation, genomic instability, and ataxia-telangiectasia-like disease [13]. Mre11 is made up of three domains, the core nuclease domain, the capping domain, and the C-terminal helix-loop-helix domain, which is also called the Rad50 binding domain (RBD). The Mre11 RBD interacts with the coiled-coil domain of Rad50 and it is thought that any allosteric communication between Rad50 and Mre11 proceeds through this interface [14]. By itself, Mre11 has poor DNA binding affinity due to an autoregulation mechanism where a negatively charged flexible linker separating the capping domain from the RBD competes with DNA for a binding site near the interface between the capping and core domains [15]. Formation of the MR complex pulls the flexible linker away
from Mre11 and reveals the high affinity DNA binding site on Mre11. Rad50 is in turn activated upon complex formation, with its ATPase activity increasing from 0.15 sec\(^{-1}\) to 3 sec\(^{-1}\). The role of ATP hydrolysis in Rad50 has been controversial. The Tainer lab has suggested that ATP binding acts as an allosteric switch to activate Mre11 nuclease activity, whereas ATP hydrolysis and product release turns it off [8]. The Paull lab has demonstrated weak Rad50-catalyzed DNA unwinding activity that is dependent on ATP hydrolysis, although this has not been observed by others [16], [17]. The Nelson lab has suggested that ATP hydrolysis is used to increase the processivity of the MR complex and drive translocation along the ssDNA product [18].

In addition to the lack of clarity surrounding the function of ATP hydrolysis in Rad50, the degree of intersubunit communication has been a source of contention. Even though all ABC proteins have similar structures, there appears to be some fundamental differences in the way they bind and hydrolyze ATP. Some ABC proteins to only use one ATP active site, whereas others use both ATP active sites [19]. In the case of Rad50, it is known that both ATP active sites are able to bind and activate ATP [20]. However, there are conflicting reports concerning the amount of coupling that occurs between the ATP active sites. A possible source of these discrepancies is that most researchers working on Rad50 do not investigate the full-length Rad50 protein. Instead, the coiled-coil/zinc-hook domains are removed for ease of expression and purification. In bacteriophage T4, removal of the coiled-coil domain has drastic effects on Rad50’s ATP hydrolysis activity and its ability to activate Mre11 [21].

The Nelson lab has been investigating allosteric coupling in Rad50 using several approaches [15], [18], [22], [23]. This report describes efforts to create and characterize a Rad50 heterodimer with a single functioning ATP site. The heterodimer was constructed by co-expressing two Rad50 genes, one containing a Histidine tag (Rad50-EP\(_{WT}\)) and the other
containing a Glutamate tag (Rad50-NX\textsubscript{WT}). Mutagenesis was used to obtain the D512A on one subunit and E505Q on the other subunit resulting in the Rad50-EP\textsubscript{D512A NX\textsubscript{E505Q}} heterodimer. It was found that in the absence of Mre11 and DNA, the heterodimer with the single ATP active site had about 50% ATPase activity compared to Rad50\textsubscript{WT}. However, when the heterodimer formed a complex with Mre11 and DNA, the activity was greatly reduced compared to the MR\textsubscript{WT} complex, suggesting that the active sites are strongly coupled and Rad50 with a single ATP filled is unable to be allosterically activated by DNA.

**Methods**

*DNA Constructs-*

To generate a single plasmid containing two Rad50 genes, we first constructed two separate Rad50 expression plasmids, one containing an N-terminal hexa-histidine tag (petDuet-Rad50EP) and the other containing an N-terminal hexa-glutamate tag (petDuet-Rad50NX). To create petDuet-Rad50NX, the Rad50 gene from pET28-Rad50 was amplified via PCR and cloned into the pET Duet-1 expression vector using the following primers, which incorporated the NdeI and XhoI restriction sites that were used for digestion and ligation: T4Rad50-NdeF: 5’-GGGAATTCCATATGAAGAATTTTAACTTAATAG-3’ and T4Rad50-XhoR: 5’-CCACCACGTGAGTTACACCATGACCGTGAATCTGC-3’.

To create petDuet-Rad50EP, the DNA sequence of the Rad50 open reading frame was altered so that the codon usage differed as much as possible from the other Rad50 open reading frame. The Rad50 gene with altered codons was then synthesized and cloned into a pUC19 vector by Genscript. The codon-altered Rad50 gene was then amplified via PCR and cloned into the pET Duet-1 expression vector using the following primers, which incorporated the EcoRI
and PstI restriction sites that were used for digestion and ligation: T4Rad50-EcoF: 5’-GGTGGTGAATTCTGATGAAAAATTCTCAATTAAAAA-3’ and T4Rad50-PstR: 5’-CCACCCTGCAGTTACACCAGTACGTGAAATCTGC-3’.

Each plasmid was then digested with PstI and EcoRI. The petDuet-Rad50EP insert was then ligated into the petDuet-Rad50NX plasmid to create petDuetEPWTNXWT. The open reading frames of both Rad50 genes were verified via DNA sequencing by the Iowa State University (ISU) DNA Facility.

*Mutagenesis of petDuetEPWTNXWT*

The D512A and E505Q mutations were incorporated into the petDuetEPWTNXWT plasmid using the Quikchange Lightning Multi Site-Directed Mutagenesis kit. The primers were designed so that they were able to anneal at the same time and ensured that the D512A primer only annealed to the His-tag (EP) gene and the E505Q primer only annealed to the Glu-tag (NX) gene. The following primers were used for mutagenesis: D512A-F: 5’-CGACGTTTCTTCTGGCCGACAGGAGCATAAAG-3’ and D512A-R: 5’-CTTTATGCCCTCTGCGGCGAAGGAACCGTC-3’ designed to anneal to the altered sequence of His-tag (EP). E505Q-F: 5’-GTACATTAATTCTTGATCAAGTTTTTGGGTATGGTTCTATTGGATGCC-3’ and E505Q-R: 5’-GGCATCAAATGACCCATCAAACACTTGATCAAGAATTAATGTAC-3’ designed to anneal to the Glu-tag (NX) gene. Sequencing provided by the ISU DNA Facility confirmed successful mutations and the plasmid construct was then named petDuetEPD512ANXE505Q
Protein Expression and Purification of Rad50-EP<sub>WT</sub>/NX<sub>WT</sub> and Rad50-EP<sub>D512A/</sub>NX<sub>E505Q</sub> Heterodimers

The petDuet-Rad50EP<sub>WT</sub>/NX<sub>WT</sub> plasmid was transformed into BL21(DE3) cells and plated on LB-agar plates containing 100μg/ml ampicillin. A single colony from the plate was then used to inoculate a 100ml flask of LB-amp and was placed in a shaker for 16 hours at 37°C. 10ml each of the 100ml culture was used to inoculate four, 1-liter flasks of LB-AMP, which were shaken at 220rpm at 37°C until they reached an OD<sub>600</sub> of 0.7. The flasks were then cooled to 16°C and expression was induced by adding 0.2mM IPTG and placed in the shaker at 220rpm at 37°C for 16 hours. The cells were then collected by centrifugation at 4000 rpm for 10 minutes, re-suspended in 1x Nickel Binding Buffer (40mM Tris-HCl pH 8, 500mM NaCl, 5mM Imidazole, 10% glycerol), and lysed using an Emulsiflex homogenizer. All of the purification steps were carried out at 4°C. The lysed cells were centrifuged at 17000 rpm for 50 minutes. The cell-free extract was loaded onto a column packed with 5ml of nickel-agarose beads at a rate of ~1-2ml/minute. The Rad50 homodimer containing two glutamate tags (Rad50-NX<sub>WT</sub>/NX<sub>WT</sub>) flows through the column at this step. The column was then washed with 100ml of binding buffer followed by a wash containing 50ml of binding buffer + 15mM imidazole (20mM imidazole final concentration). The third wash was 100ml binding buffer + 0.5M NaCl (1M NaCl final concentration) followed by a fourth wash of 25ml containing 200mM NaCl, 20mM Tris pH 7.6, and 10% glycerol. The protein was eluted off using the nickel elution buffer (200mM NaCl, 20mM Tris pH 7.6, 10% glycerol, and 150mM Imidazole). The elution contains a mixture of Rad50 homodimer with two histidine tags (Rad50-EP<sub>WT</sub>/EP<sub>WT</sub>) and a Rad50 heterodimer containing single histidine and glutamate tags (Rad50-EP<sub>WT</sub>/NX<sub>WT</sub>). This elution was then dialyzed overnight in a 2L dialysis buffer (200mM NaCl, 20mM Tris pH 7.6, and 20%
glycerol). The protein was removed from the dialysis buffer and loaded onto a sulfopropyl-Sepharose (SP-Sepharose) column at a flow rate of 1ml/minute. The heterodimeric protein containing a single histidine tag and a single glutamate tag (Rad50-EPWT/NXWT) does not bind to SP-sepharose and therefore flowed through the column. The column was washed with 200mM NaCl, 20mM Tris pH 7.6, 10% glycerol and the Rad50 dimer with two histidine tags (Rad50-EPWT/EPWT) was eluted with 400mM NaCl, 20mM Tris pH 8, and 20% glycerol. All samples were run on SDS-PAGE gel to analyze purity. The SDS-PAGE buffer contained 25mM 2-Amino-2-hydroxymethyl-propane-1,3-diol (TRIS), 192mM Glycine, and 0.1% Sodium dodecyl sulfate (SDS). WT-Rad50 (His/His), WT-Rad50 (Glu/Glu), and Rad50-EPWT/NXWT were also run on a 0.6% neutral agarose gel for further confirmation. The neutral agarose gel was made with a buffer containing 25mM Tris-Acetate pH 8 and 19.2mM glycine. Protein was concentrated to ~100µM, frozen in small aliquots with liquid nitrogen, and stored at -80°C. The extinction coefficient for determining the protein concentration of T4 Rad50 is 33,140 cm⁻¹ M⁻¹.

The Rad50-EPD512A/NXE50Q heterodimer was expressed and purified in an identical fashion as Rad50-EPWT/NXWT.

DNA Substrates-

The 50-bp dsDNA substrate needed for the ATPase kinetics was prepared by annealing two complementary oligonucleotides that had the following sequences: ds50-merTop: 5’-CTCTTGGTAGTTATGAGTTGGAATACATTTTATTTCCATTATCAATTAG-3’ and ds50-merBottom: 5’-CTAATTGATAATTAAATGTATTGCACACCATCATCATAATCACC AAGAG-3’. The DNA substrates needed for the nuclease assays were a 1 position and 18 position, relative to the 3’ end, 2-aminopurine (2-AP) substrate. The 1 position 2-AP DNA
substrate had an identical sequence as the ds50-merTop except the sequence had the 3’ G replaced with 2-AP. The 18 position 2-AP DNA substrate was an identical sequence to the ds50-merTop with the adenine at position 18 replace with 2-AP. The ds50-merBottom for both 1 and 18 position DNA substrates was identical to the sequence used previously.

**Steady-state ATPase Kinetics**

To determine the steady-state ATP hydrolysis activity of Rad50 and the MR complex we used a standard fluorometric coupling assay. ADP formation was coupled to NADH consumption through the enzymatic action of pyruvate kinase and lactate dehydrogenase. All assays were carried out at 30°C using excitation wavelength of 340nm and emission wavelength of 460nm on a Cary Eclipse spectrofluorometer. The assay buffer contained 50mM Tris-HCl pH 7.6, 50mM KCl, 5mM MgCl₂, 100µM phosphoenolpyruvate, 6.67 units/ml pyruvate kinase, 10units/ml lactate dehydrogenase, and 0.1mg/ml BSA. Adding Rad50 (1µM final concentration per reaction) or the preassembled MR complex (0.25µM Mre11 WT/0.2µM Rad50 final concentration per reaction) initiated the ATPase reactions. Initial velocity (v) was measured at different ATP concentrations ([S]) and the maximum velocity (Vₘₐₓ), Michaelis constant (Kₘ), and Hill coefficient (n) were estimated with SigmaPlot.

**Determination of Nuclease Activity**

The nuclease activity was determined spectrofluorometrically using DNA substrates with containing a fluorescent 2-aminopurine deoxyribonucleotides (2-AP) at the 1st or 18th position relative to the 3’ end of the substrate. When the 2-AP is excised from the DNA through the nuclease activity of Mre11 the fluorescent signal increases. The assay was carried out at 30°C
using excitation wavelength of 310nm and emission wavelength of 375nm on a Cary Eclipse spectrofluorometer. The assay buffer contained 50mM Tris-HCl, 50mM KCl, 5mM MgCl₂, 0.3mM MnCl₂ and 1.3µM DNA substrate. For the 1ˢᵗ position substrate the concentrations of Mre11 and Rad50 were 50 and 55nM, respectively. For the 1⁸ᵗ position substrate the concentrations of Mre11 and Rad50 were 400 and 440nM, respectively. When added to the reactions, the concentration of ATP was 1mM.

**Tryptophan Quenching Assay**

Tryptophan quenching was determined using increasing concentrations of ATP and measuring fluorescence and absorbance at each concentration. The assay buffer contained 50mM Tris-HCl pH 7.6, 50mM KCl, 150 mM NaCl, 5mM MgCl₂, and 1µM of protein. The titration was carried out at room temperature on a Cary Eclipse spectrofluorometer. The PMT was set to a high voltage (800mV), the excitation and emission wavelengths were 295 and 340 nm, respectively, and average time of acquisition was 1 second. The absorbance at 295nm was also determined for each concentration using a Cary 50 UV-Vis Spectrophotometer. This value was then used to correct for the inner-filter effect using the following equation:

\[
F_{corr} = F_0 \times 10^{(A_{295}/2)} \quad (\text{Eq. 1})
\]

**Results**

**Expression and Purification**

Approximately 20 mg of Rad50-EP<sub>WT</sub>/EP<sub>WT</sub> plus Rad50-EP<sub>WT</sub>/NX<sub>WT</sub> protein eluted from the nickel-agarose column elution and 9.5 mg of Rad50-EP<sub>WT</sub>/NX<sub>WT</sub> flowed through the SP-Sepharose column. Small aliquots were collected from each purification step and run on a SDS-
PAGE gel. Approximately 4μg of each sample were separated using 10% SDS-PAGE and stained with Coomassie Blue R-250 (Figure 1). The gel shows successful purification of Rad50-EPWT/NXWT and the neutral agarose gel (Figure 2) suggests that we obtained the Rad50-EPWT/NXWT heterodimer.

The EP<sub>D512A/NXE505Q</sub> heterodimer expressed at a similar level as WT in the original pET28 vector. The nickel-agarose column elution was ~30mg and an overall yield after purification (SP-Sepharose column flow through) was ~8mg. Approximately 4μg of each sample were separated using 10% SDS-PAGE and stained with Coomassie Blue R-250 shows successful purification of the Rad50-EP<sub>D512A/NXE505Q</sub> heterodimer (Figure 3).

**Steady State ATP Hydrolysis Activity of Rad50-**

Time courses of ATP hydrolysis by Rad50 were collected for increasing amounts of ATP (5-3200μM). The initial velocities were determined from the linear portion of the time course and the slopes of the lines were converted to μM ADP·s<sup>-1</sup> per second. The data points were then fit to the Hill Equation using SigmaPlot.

\[
    v = \frac{V_{\text{max}}[S]^n}{K_m^n + [S]^n}
\]

(Eq. 2)

In the Hill equation \(v\) is velocity, \(K_m\) (μM) is the Michaelis constant, \(V_{\text{max}}\) is the maximum velocity at saturated substrate concentration, and \(n\) is the Hill coefficient. The fitted data yielded a \(k_{\text{cat}}\) of 0.145 ± 0.003 s<sup>-1</sup> for Rad50<sub>WT</sub> and a \(k_{\text{cat}}\) of 0.0791 ± 0.0171 s<sup>-1</sup> for Rad50-EP<sub>D512A/NXE505Q</sub> heterodimer (Table 1). Both single mutants Rad50-EP<sub>D512A/EPD512A</sub> and Rad50-NXE505Q/NXE505Q have significantly lower \(k_{\text{cat}}\) values of 0.0037 ± 0.0003 s<sup>-1</sup> and 0.005 ± 0.0002 s<sup>-1</sup> respectively. This strongly suggests that we have obtained a heterodimer with a single functioning ATP active site. Rad50<sub>WT</sub> has a \(K_m\)-ATP value of 16μM and the Rad50-
EP_{D512A/\text{N}X_{E505Q}} heterodimer has a $K_m$-ATP value of 1120$\mu$M. The elevated $K_m$-ATP suggests that the ‘wild-type’ active site has a reduced affinity for ATP as compared to the Rad50\text{WT} homodimer. The Hill coefficient for the Rad50-EP_{D512A/\text{N}X_{E505Q}} heterodimer was reduced from 1.4 for the Rad50\text{WT} homodimer to 0.9, which is consistent with a single ATPase active site. Due to the very low activity of the E505Q and D512A homodimers, the $K_m$-ATP and Hill coefficients could not be determined.

*Steady State ATP Hydrolysis Activity of the MR complex*

Time courses of ATP hydrolysis by the MR complex were collected for increasing amounts of ATP (5-3200$\mu$M) + 1$\mu$M dsDNA and the data was fit to the Hill Equation (eq. 2) using SigmaPlot. The data fit yielded a $k_{cat}$ of 3.2 ± 0.1 s$^{-1}$ for the MR\text{WT} complex and a $k_{cat}$ of 0.1205 ± 0.0131 s$^{-1}$ for the MR-EP_{D512A/\text{N}X_{E505Q}} heterodimer complex (Table 2). The single mutants Rad50-EP_{D512A/\text{E}P_{D512A}} and Rad50-\text{N}X_{E505Q}/\text{N}X_{E505Q} have a value of 0.027 ± 0.002 s$^{-1}$ and 0.0103 ± 0.0007 s$^{-1}$ respectively. This again confirms that we have a heterodimer because the $k_{cat}$ is much larger than the single mutants. MR\text{WT} has a $K_m$-ATP value of 49$\mu$M and a Hill coefficient of 2.4. However, the MR-EP_{D512A/\text{N}X_{E505Q}} heterodimer has a $K_m$-ATP value of 658$\mu$M and a Hill coefficient of 1.1. The single mutants Rad50-EP_{D512A/\text{E}P_{D512A}} and Rad50-\text{N}X_{E505Q}/\text{N}X_{E505Q} have $K_m$-ATP values of 0.027$\mu$M and 0.0103$\mu$M respectively and a Hill coefficient of 1.5 and 1.1 respectively.

*Nuclease Activity*

The initial nuclease activity of the MR complex can be analyzed with a DNA substrate containing a fluorescent 2-AP at the 1$^{st}$ position relative to the 3’ end of the substrate. Mre11
alone has a nuclease activity of \(0.319 \pm 0.039\text{s}^{-1}\) and the nuclease activity of \(\text{MR}_{\text{WT}}\) is \(6.766 \pm 0.107\text{s}^{-1}\) (Table 3). \(\text{MR-NX}_{\text{E505Q}}/\text{NX}_{\text{E505Q}}\) and \(\text{MR-EP}_{\text{D512A}}/\text{EP}_{\text{D512A}}\) homodimers have a nuclease activity of \(3.366 \pm 0.144\text{s}^{-1}\) and \(0.883 \pm 0.019\text{s}^{-1}\) respectively. The \(\text{MR-EP}_{\text{D512A}}/\text{NX}_{\text{E505Q}}\) heterodimer had 1-position nuclease activity of \(1.894 \pm 0.113\text{s}^{-1}\), which demonstrates that the Rad50 heterodimer is capable of forming a complex with Mre11 and suggests that its DNA binding activity is unaffected. The 18 position 2-AP resulted in \(\text{MR}_{\text{WT}}\) having a nuclease activity of \(0.0089\text{s}^{-1}\) without ATP and \(0.057\text{s}^{-1}\) with ATP (Table 4). The 6.4 fold increase in 18 position nuclease activity suggests that ATP is being utilized to increase the processivity and translocation rate of the complex [22]. \(\text{MR-NX}_{\text{E505Q}}/\text{NX}_{\text{E505Q}}\) has a nuclease activity of \(0.080 \pm 0.044\text{s}^{-1}\) with ATP and \(\text{MR-EP}_{\text{D512A}}/\text{EP}_{\text{D512A}}\) has a nuclease activity of \(0.04 \pm 0.003\text{s}^{-1}\) without ATP and \(0.006 \pm 0.0005\text{s}^{-1}\) with ATP. The \(\text{MR-EP}_{\text{D512A}}/\text{NX}_{\text{E505Q}}\) heterodimer has a nuclease activity of \(0.0061 \pm 0.002\text{s}^{-1}\) without ATP and \(0.0019 \pm 0.0003\text{s}^{-1}\) with ATP. The 3.2 fold decrease in nuclease activity in the presence of ATP suggests that the complex is “stuck” on its product and is unable to dissociate or translocate to the next nucleotide [18].

**Tryptophan Quenching**

The tryptophan quenching assay was conducted in order to determine the \(K_{d-\text{ATP}}\) (\(\mu\text{M}\)) for the ATPase activity of \(\text{Rad50}_{\text{WT}}\) and the \(\text{Rad50-EP}_{\text{D512A}}/\text{NX}_{\text{E505Q}}\) heterodimer. Increased amounts of ATP were titrated into the cuvette and tryptophan fluorescence at 340 nm was read at each concentration. The data that was collected was fitted using equation 3 with SigmaPlot.

\[
f = \frac{(100-c)}{1+(\frac{x}{K_d})^n+c}
\]

(Eq. 3)
The results showed that the $K_d$ ($\mu$M) of the $\text{Rad50-EP}_{D512A}/\text{NX}_{E505Q}$ heterodimer is 90.7 ± 14 $\mu$M, whereas the $K_d$-ATP for $\text{Rad50}_{\text{WT}}$ is 9.4 ± 1.2 $\mu$M (Table 1). The percent of normalized fluorescence for each of these proteins is shown in Figure 4. Interestingly, while the maximum level of fluorescent quenching of $\text{Rad50}_{\text{WT}}$ is approximately 40%, the tryptophan fluorescence $\text{Rad50-EP}_{D512A}/\text{NX}_{E505Q}$ is only quenched 20%.

**Discussion**

The role of ATP hydrolysis in Rad50 function has been the subject of much debate. Several possible models exist for what ATP hydrolysis might be doing, from acting as an on/off switch, to powering dsDNA unwinding, to driving processive translocation. In the latter two processes Rad50 acts as a motor, which should require strong coupling between active ATPase sites, whereas the switch model could be carried out with only a single functioning ATP site. Indeed, some ABC transporter proteins show half-of-sites reactivity where a single ATP site acts to open and close the channel for membrane transport.

We set out to determine the effect of eliminating just one of the two Rad50 ATPase active sites. If the ATP active sites are tightly coupled, then we would expect ATP hydrolysis to be severely diminished in the single ATP site heterodimer. On the other hand, if the ATP sites function in an independent fashion or display half-of-sites reactivity, then the ATPase activity of the heterodimer is expected to be either half or the full value of the WT enzyme, respectively.

We found that the Rad50 heterodimer with a single functioning ATP site retained approximately half the activity of the fully WT Rad50 homodimer. This is a strong indication that both ATP sites operate in the WT protein, but that the two active sites are uncoupled and not in communication with each other. In contrast to Rad50 alone, when bound to Mre11 and
dsDNA, the single ATP site heterodimer has much less activity than the two active site homodimer (26-fold decrease). In fact, it would appear that dsDNA no longer activates the single ATP site MR complex (only 1.5-fold increase in activity).

In terms of allostery, dsDNA can be considered a V-type allosteric activator of the MR complex, increasing its $k_{\text{cat}}$-ATP but not affecting its $K_m$-ATP [15]. The rate-limiting step of Rad50, the MR complex, and the MR-DNA complex is the hydrolysis step (i.e., chemistry), suggesting that binding of dsDNA to the MR$_{\text{WT}}$ complex causes a conformational change in the ATP active sites that brings a catalytic residue(s) into a more favorable position for catalysis [18]. The lack of dsDNA activation in the single ATP site heterodimer suggests that both ATP sites need to be occupied for dsDNA to exert its effect on catalysis. The requirement for both ATP sites to be filled to achieve maximal rate enhancement would result in sigmoidal initial rate versus ATP concentration curves, as we observe for the MR$_{\text{WT}}$-DNA complex. This type of allostery is a kinetic phenomenon and should not be displayed in equilibrium binding assays, since the activation is restricted to an increase in $k_{\text{cat}}$, whereas the $K_m$-ATP is unchanged. Consistent with this, the $K_d$-ATP values determined using tryptophan fluorescence fit well to simple binding equations with Hill coefficients of 1.0. A summary of this model is shown in Figure 5.
References


Tables and Figures

**Table 1**

*Steady State ATPase Kinetic Constants for WT and Mutant Rad50*

<table>
<thead>
<tr>
<th>Protein</th>
<th>$k_{\text{cat}}$ (s^{-1})</th>
<th>K_m-ATP (µM)</th>
<th>Hill coefficient</th>
<th>K_d (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT(^a)</td>
<td>0.145 ± 0.003</td>
<td>16 ± 1</td>
<td>1.4 ± 0.1</td>
<td>9.4 ± 1.2</td>
</tr>
<tr>
<td>E505Q(^a)</td>
<td>0.005 ± 0.002</td>
<td>ND</td>
<td>ND</td>
<td>5.6 ± 0.6</td>
</tr>
<tr>
<td>D512A(^b)</td>
<td>0.0037 ± 0.0003</td>
<td>ND</td>
<td>ND</td>
<td>220 ± 24</td>
</tr>
<tr>
<td>D512A/E505Q</td>
<td>0.0791 ± 0.0171</td>
<td>1130± 624</td>
<td>0.9 ± 0.2</td>
<td>90.7 ± 14</td>
</tr>
</tbody>
</table>


Table 2

*Steady State ATPase Kinetic Constants for WT and Mutant Rad50 in the Presence of Mre11 and DNA*

<table>
<thead>
<tr>
<th>Protein</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>$K_m$-ATP (µM)</th>
<th>Hill coefficient</th>
<th>Mre11/DNA activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT$^a$</td>
<td>3.2 ± 0.1</td>
<td>49 ± 2</td>
<td>2.4 ± 0.2</td>
<td>21.9</td>
</tr>
<tr>
<td>E505Q$^a$</td>
<td>0.0103 ± 0.0007</td>
<td>27 ± 1</td>
<td>1.1 ± 0.2</td>
<td>2.6</td>
</tr>
<tr>
<td>D512A$^b$</td>
<td>0.027 ± 0.002</td>
<td>410 ± 51</td>
<td>1.5 ± 0.2</td>
<td>7.3</td>
</tr>
<tr>
<td>D512A/E505Q</td>
<td>0.1205 ± 0.0131</td>
<td>658 ± 404</td>
<td>1.1 ± 0.5</td>
<td>1.5</td>
</tr>
</tbody>
</table>


### Table 3

*Nuclease Activity in the 2-aminopurine 1 position*

<table>
<thead>
<tr>
<th>Protein</th>
<th>Nuclease Activity (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mre11</td>
<td>0.319 ± 0.039</td>
</tr>
<tr>
<td>Mre11-Rad50&lt;sub&gt;WT/WT&lt;/sub&gt;</td>
<td>6.766 ± 0.107</td>
</tr>
<tr>
<td>Mre11-Rad50&lt;sub&gt;E505Q/E505Q&lt;/sub&gt;</td>
<td>3.366 ± 0.144</td>
</tr>
<tr>
<td>Mre11-Rad50&lt;sub&gt;D512A/D512A&lt;/sub&gt;</td>
<td>0.883 ± 0.019</td>
</tr>
<tr>
<td>Mre11-Rad50&lt;sub&gt;D512A-E505Q/WT&lt;/sub&gt;</td>
<td>1.894 ± 0.113</td>
</tr>
</tbody>
</table>
Table 4

*Nuclease Activity in the 2-aminopurine 17 position*

<table>
<thead>
<tr>
<th>Protein</th>
<th>Nuclease Activity (s&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Nuclease Activity (+ATP) (s&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.0089</td>
<td>0.0570</td>
</tr>
<tr>
<td>E505Q&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND</td>
<td>0.080 ± 0.044</td>
</tr>
<tr>
<td>D512A&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.04 ± 0.003</td>
<td>0.006 ± 0.0005</td>
</tr>
<tr>
<td>D512A/E505Q</td>
<td>0.0061 ± 0.002</td>
<td>0.0019 ± 0.0003</td>
</tr>
</tbody>
</table>


Figure 1. **SDS-PAGE gel of Rad50 EPNX WT heterodimer purification.** 1- protein ladder, 2- total cell lysate, 3- cell-free extract, 4- nickel-agarose column flow through, 5- nickel-agarose column elution, 6- Sp Sepharose column flow through, 7- Sp Sepharose column elution. This shows that the Rad50-NX<sub>WT</sub>/NX<sub>WT</sub> dimer flowed off the nickel column and the Rad50-E<sub>WT</sub>P<sub>WT</sub>/E<sub>WT</sub>P<sub>WT</sub> dimer and Rad50-E<sub>WT</sub>P<sub>WT</sub>/NX<sub>WT</sub> heterodimer were eluted off. The nickel elution was loaded onto the Sp Sepharose column, which resulted in Rad50-E<sub>WT</sub>P<sub>WT</sub>/NX<sub>WT</sub> heterodimer flowing off and the Rad50-E<sub>WT</sub>P<sub>WT</sub>/E<sub>WT</sub>P<sub>WT</sub> dimer was eluted off the column.
Figure 2. **Neutral agarose gel of wild type Rad50, Rad50 EPNX WT heterodimer, and pET16 WT.** 1- WT-Rad50 (His/His) (30µg), 2- WT-Rad50 (His/His) (40µg), 3- Rad50-EP<sub>WT</sub>/NX<sub>WT</sub> heterodimer (20µg), 4- Rad50-EP<sub>WT</sub>/NX<sub>WT</sub> heterodimer (40µg), 5- WT-Rad50 (Glu/Glu) (40µg), 6- WT-Rad50 (Glu/Glu) (60µg). The samples were run on a 0.6% agarose gel and each sample had two different concentrations. The buffer contained 25mM Tris-Acetate pH 8 and 19.2mM glycine. The gel was run for 3 hours at 50V and then it was stained and de-stained for analysis. This confirms that the Rad50 EPNX WT heterodimer was successfully obtained.
Figure 3. **SDS-PAGE gel of D512A/E505Q heterodimer purification.** 1- protein ladder, 2- total cell lysate, 3- cell-free extract, 4- nickel-agarose column flow through, 5- nickel-agarose column elution, 6- Sp Sepharose column flow through, 7- Sp Sepharose column elution. This shows that the Rad50-NX<sub>E505Q</sub>/NX<sub>E505Q</sub> dimer flowed off the nickel column and the Rad50-EP<sub>D512A</sub>/EP<sub>D512A</sub> and Rad50-EP<sub>D512A</sub>/NX<sub>E505Q</sub> heterodimer were eluted off. The nickel elution was loaded onto the Sp Sepharose column, which resulted in Rad50-EP<sub>D512A</sub>/NX<sub>E505Q</sub> heterodimer flowing off and the Rad50-EP<sub>D512A</sub>/EP<sub>D512A</sub> dimer was eluted off the column.
Figure 4. **Tryptophan quenching.** Normalized fluorescence (%) at increasing amounts of ATP (0-500µM) for Rad50 WT and Rad50 D512A/E505Q heterodimer.
Figure 5. **Model for allosteric activation of the MR complex in the presence of DNA.** In the absence of DNA the catalytic residues in the ATP active site are in suboptimal positions and the Rad50 dimer behaves as two independent subunits, which hydrolyze ATP in an uncoordinated fashion. The binding of DNA converts Rad50 into an allosteric enzyme that follows a V-type kinetic pattern (enhancement of catalytic activity rather than affinity). However, ATP must bind to both active sites for the dsDNA-induced conformational change that enhances catalytic activity. This leads to kinetic ATPase cooperativity (sigmoidicity in velocity vs. ATP plots) but not ATP binding cooperativity.
CHAPTER III: CONCLUSIONS

Summary

The specific role of Rad50 ATP hydrolysis is unknown because there is a debate on whether or not it is necessary for coupling of ATP active sites in order to carry out its proper function. Therefore, we wanted to investigate the activity of a Rad50 heterodimer containing a single ATP active site. By generating and characterizing the single ATPase heterodimer, we were able to show that Rad50 alone has independent ATP binding sites. This is evident because the $k_{cat}$ for Rad50-EP$_{D512A/NX_{E505Q}}$ heterodimer was about 50% of the Rad50$_{WT}$ protein. However, in the presence of Mre11, the ATP active sites appear to become tightly coupled such that the MR-EP$_{D512A/NX_{E505Q}}$ heterodimer activity is significantly lower than MR$_{WT}$. Moreover, the ability of the MR-EP$_{D512A/NX_{E505Q}}$ complex to perform multiple nucleotide excisions along a DNA substrate was severely diminished.

The hydrolysis step of Rad50, the MR complex, and the MR-DNA complex is rate-determining, which suggests that the activation by dsDNA is because of a conformational change in the ATP active sites that results from dsDNA binding to the MR$_{WT}$ complex. The single ATP active site had very low dsDNA activation compared to the MR$_{WT}$ complex, suggesting that both of the ATP active sites must be occupied in order for dsDNA to carry out its activation function.

Future Directions

The $K_m$-ATP was much higher for the Rad50-EP$_{D512A/NX_{E505Q}}$ heterodimer as compared to Rad50$_{WT}$, which might suggest that the double-mutant active site has partially disrupted the dimer interface and these structural changes have propagated to the functioning active site. To
test for this possibility, we need to find a new approach to creating a single ATP active site heterodimer. Instead of using both the E505Q and D512A mutations, the heterodimer can be made with just one of these mutations. Using a single mutation on one subunit and the ‘wild type’ on the other subunit will still create a heterodimer with a single ATP active site because the mutation will kill one of the active sites. However, we must be more careful to fully separate the heterodimer from the homodimer, as any contamination by WT will make our activity measurements artificially high. This problem can be alleviated by placing the WT Rad50 gene in petDuetNX, since in that case the WT protein should not bind to the nickel-agarose column. The E505Q homodimer has a low Km and Kd-ATP, but poor hydrolysis activity. It is possible that a heterodimer with that mutation will be fully activated by ATP, because both ATP sites will be occupied. On the other hand, it is also possible that ATP hydrolysis will be completely shut-down, if unhydrolyzed ATP in the non-functional active site prevents release of products in the functional active site.

It will also be interesting to perform hydrogen-deuterium (HD) exchange mass spectrometry on the singly bound ATP protein to see if only half of the structure changes in the presence of ATP and dsDNA compared to the Rad50_{WT} homodimer. If so, then this would help confirm our model presented in Figure 5 of chapter 2.

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