Crystal Structure of an ATPase-active Form of Rad51 Homolog from Methanococcus voltae

INSIGHTS INTO POTASSIUM DEPENDENCE*

Received for publication, September 27, 2004, and in revised form, October 27, 2004
Published, JBC Papers in Press, November 10, 2004, DOI 10.1074/jbc.M411093200

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Homologous gene recombination is crucial for the repair of DNA. A superfamily of recombinases facilitate a central strand exchange reaction in the repair process. This reaction is initiated by coating single-stranded DNA (ssDNA) with recombinases in the presence of ATP and Mg²⁺ co-factors to form helical nucleoprotein filaments with elevated ATPase and strand invasion activities (1). At the amino acid sequence level, archaeal RadA and Rad51 and eukaryal Rad51 and meiosis-specific DMC1 form a closely related group of recombinases distinct from bacterial RecA (2). Unlike the extensively studied Escherichia coli RecA (EcRecA), increasing evidences on yeast and human recombinases imply that their optimal activities are dependent on the presence of a monovalent cation, particularly potassium (3–5). Here we present the finding that archaeal RadA from Methanococcus voltae (MvRadA) is a stringent potassium-dependent ATPase, and the crystal structure of this protein in complex with the non-hydrolyzable ATP analog adenosine 5′-(β,γ-iminotriphosphate), Mg²⁺, and K⁺ at 2.4 Å resolution. Potassium triggered an in situ conformational change in the ssDNA-binding L2 region concerted with incorporation of two potassium ions at the ATPase site in the RadA crystals preformed in K⁺-free medium. Both potassium ions were observed in contact with the γ-phosphate of the ATP analog, implying a direct role by the monovalent cations in stimulating the ATPase activity. Cross-talk between the ATPase site and the ssDNA-binding L2 region visualized in the MvRadA structure provides an explanation to the co-factor-induced allosteric effect on RecA-like recombinases.

Homologous gene recombination enables repair of double-stranded DNA breaks or stalled replication forks (6–10) using another DNA molecule as the template in a diploid or replicating cell. This process involves a pivotal DNA strand exchange reaction facilitated by a recombinase superfamily (11) composed of bacterial RecA (12), archaeal RadA or Rad51 (13), and eukaryal Rad51 (14) and meiosis-specific DMC1 (15). In Escherichia coli, a double-stranded break is first processed by asymmetric unwinding and exonuclease digestion to produce a long stretch of ssDNA (16, 17). In the presence of ATP and Mg²⁺, the recombinase coats this primary ssDNA to form a nucleoprotein filament with ATPase and strand exchange activities (1). Strand exchange enables replication using the identical sister chromatid or a homologous chromosome as the template. Homologous recombination in meiosis generates crossover of homologous chromosomes.

At the tertiary structure level, all RecA-like recombinases share an ATPase domain preceded by a short β-stranded polymerization motif (18, 19) (Fig. 1, PM). Their respective N- and C-terminal domains implicated for binding dsDNA (20, 21), however, vary. Rad51, DMC1, and RadA possess a similar-sized domain at its C terminus (22). Distinctive from bacterial RecA, archaeal and eukaryal recombinases form a closely related group with a typical identity level of ~40% (2). The bacterial RecA recombinases have only ~20% identity to the non-bacterial recombinases (2).

Despite weak amino acid sequence similarity among the RecA/Rad51/DMC1/RadA superfamily, documented results have revealed a common tale of two right-handed helical structures: an extended “active” form and a compact “inactive” form. Recently, yeast Rad51 and MvRadA have been crystallized in the extended filamentous form (23, 24). In both structures, the ATPase sites were placed between protomers, a location resembling that of the electron microscopy-reconstructed active filament of EcRecA (25). As such, all the RecA-like recombinases likely share the structural characteristics of an active extended nucleoprotein filament in the presence of three known co-factors: ssDNA, ATP, and Mg²⁺. Comparative in vitro studies, however, have shown that optimal reaction conditions are different (3–5). EcRecA favors low salt conditions with little or no monovalent cation. Human and yeast Rad51 and DMC1, on the other hand, favor the presence of a salt, typically KCl or (NH₄)₂SO₄. To find an intuitive explanation of the differences in ion dependence, we studied the K⁺-dependent ATPase activity of MvRadA and determined the crystal structure of MvRadA at 2.4 Å resolution in the presence of an activating dose of KCl.

EXPERIMENTAL PROCEDURES

Protein Preparation and Crystallization—RadA from Methanococcus voltae was subcloned into pET28a, overexpressed, and purified as reported (24). The protein (~30 mg/ml) was crystallized using the hanging drop crystallization method at a room temperature of 21 °C. The optimal well solution contains 2 mM AMP-PNP (Sigma), 0.2 M MgCl₂, 6% poly-

* This work was supported in part by the Natural Sciences and Engineering Research Council of Canada, the Saskatchewan Health Research Foundation (SHRF), and the Canadian Institute of Health Research (CIHR) (all to Y. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The atomic coordinates and structure factors (code 1XU4) 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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1 The abbreviations used are: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; AMP-PNP, adenosine 5′-(β,γ-iminotriphosphate); PDB, Protein Data Bank; ATP-γ-S, adenosine 5′-O-(thiotriphosphate).
ethylene glycol 3350 (Sigma), and 0.1 M Tris-HCl at pH 7.5. The potassium form of crystals were prepared by a 10-min incubation of crystals in a solution containing 2 mM AMP-PNP, 50 mM MgCl₂, 500 mM KCl, 6% polyethylene glycol 3350, 25% glycerol, and 0.1 M Tris-HCl at pH 7.5.

**Data Collection and Structure Determination**—Potassium-soaked crystals were flash-cooled to 100 K with an Oxford Cryosystem device. The diffraction data were acquired and processed as described (24). The potassium-absent model (PDB entry 1T4G) was used as the starting
model. The entire model was iteratively rebuilt using XcalView (26) and refined using CNS (27). A 2.8-Å resolution anomalous difference map was generated using model phases retarded by 90 degrees. Two ordered potassium sites were found at 9 and $\frac{7}{2}$ peaks in this map. Lower peaks (3–4 $\frac{7}{2}$) were co-localized with sulfur atoms in Met residues. Statistics of the diffraction data, refinement, and geometry are shown in Table I.

The molecular figures are generated using Molscript (28) and Raster3D (29). The electrostatic surface is generated using GRASP (30).

**RESULTS**

**Potassium-dependent ATPase Activity of MvRadA—**Archaeal RadA recombinases (or Rad51) are feasible prototypes of eukaryal recombinases due to their ~40% sequence identity. We first examined whether MvRadA is dependent on potassium or other common monovalent cations. The reported ssDNA-dependent ATPase turnover rate was 1.2 min$^{-1}$ at a condition with 1 mM MgCl$_2$ and 25 mM KCl (32). During the optimization of reaction conditions, we observed that higher concentrations for both salts are required for optimal poly(dT)$_{36}$-dependent ATPase activity —15-fold greater than reported (Fig. 2, A and B). The ATPase assays were repeated in the presence of poly(dT-dA-dC)$_{12}$. The optimal activity on poly(dT-dA-dC)$_{12}$ was essentially identical to that on poly(dT)$_{36}$ (data not shown). More than 1 mM phosphate was detected in 30 min. In contrast, ATPase assays in varied amounts of NaCl showed no detectable activity above basal level (Fig. 2B). In assays using 10-fold elevated concentrations of enzyme and ssDNA substrate, we observed that the released phosphate in 30 min was less than 0.05 mM phosphate, which corresponded to the detection limit of absorbance 0.1 at 620 nm. Potassium is therefore estimated to be at least 200-fold more effective than sodium in stimulating the ATPase activity of MvRadA. The effects of LiCl, NH$_4$Cl, RbCl, and CsCl were essentially identical to that of NaCl (data not shown). As such, MvRadA is a stringent K$^+$-dependent ATPase.

**Structural Basis for Potassium Dependence—**EcRecA, as well as yeast and human Rad51, are known to become ATPase-active in the presence of high salt as a substitute for ssDNA (5, 33, 34). As expected, MvRadA share this property (Fig. 2B). In response, we performed an in situ experiment by soaking the crystals grown in K$^+$-free medium containing AMP-PNP and Mg$^{2+}$ (24) in a solution supplemented with 0.5M KCl, in which optimal ATPase activity was observed. Compared with unsoaked crystals (PDB entry 1T4G), the space group remained P6$_1$2$_1$2$_1$. The filament pitch coinciding with the crystallographic c axis, however, shortened from 106.7 to 104.8 Å (Table I and Fig. 3A). There were no noticeable changes in subunit orienta-

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**FIG. 2.** K$^+$-dependent ATPase activity and conformational change of MvRadA. A, ATPase activity in 100 mM KCl and varied concentration of MgCl$_2$. Half-activation was observed at 4 mM MgCl$_2$. B, ATPase activity in 10 mM MgCl$_2$ and varied concentration of KCl or NaCl. Error bars were too small to show for activities in NaCl. C, ATPase site conformation in K$^+$-free medium. D, ATPase site conformation in K$^+$-rich medium. Two MvRadA subunits are colored in yellow and gray, respectively. Two K$^+$ ions (KA and KB), one Mg$^{2+}$ ion, and water molecules are colored in purple, red, and green, respectively. The region of conformational change is circled.
tion. In both structures, the non-hydrolysable ATP analog is buried between MvRadA protomers (Fig. 3A). One subunit binds the ATP analog and an octahedral Mg\(^{2+}\) largely through its conserved P-loop (35) and the base-stacking Arg-158 (yellow subunit, Fig. 2, C and D). The other subunit contributes a region we denote the ATP cap (residues Asp-302 to Asp-308), disordered in the K\(^{-}\)rich medium. The conformational changes involve the formation of a short helix (residues Gly-275 to Ala-282). Repositioned His-280 side chain therein was observed to form a direct hydrogen bond with the γ-phosphate of the ATP analog. Additionally, a concerted conformational change of the Arg-285/Phe-107 cation-π pair partially vacated the γ-phosphate-binding site for the incorporation of two K\(^{-}\)ions. Both K\(^{-}\)ions (purple spheres, Fig. 2D) bridge the γ-phosphate with the backbone carbonyl moieties in the C terminus of the 8-residue helix. One of the K\(^{-}\)ions contacts the side chain of Asp-302, while the other contacts the side chain of glu-151. A candidate for the hydrolyzing water was also visible (large green sphere, Fig. 2, C and D). This water molecule is hydrogen bonded with the side chains of glu-151 and glu-257 from the subunit contributing the P-loop. The analogous residues of EcRecA (Glu-96 and Glu-194) have been proposed as the catalytic residues based on the EcRecA crystal structure (36) and analogy to GTPases (37). As expected for P-loop-containing ATPases and GTPases, the γ-phosphate contacts the ε-amino group of Lys-111 and the Mg\(^{2+}\) ion. Along with the added electron-withdrawing effects by His-280 and the two K\(^{-}\)ions, the γ-phosphate is likely further polarized for the nucleophilic attack by the hydrolyzing water. Thus, the K\(^{-}\)dependence by this archaean RecA orthologs is rationalized. The potassium-oxygen distances in the MvRadA structure were observed between 2.6 and 2.9 Å, resembling the values revealed in the high resolution structure of a potassium-channel-Fab complex (38). The extensive ATP-bridged interface (over 2000 Å\(^{2}\) of buried surface) (24) may have been evolved to optimally utilize the physiologically abundant potassium. Smaller cations may not be adequately coordinated by oxygen ligands. On the other hand, larger cations may not fit into the pocket without disrupting intersubunit interactions.

**DISCUSSION**

Crystal structures of EcRecA filaments (PDB entry 2REB) (36) and yeast Rad51 filaments (PDB entry 1SZP) (23) have been reported. Like all P-loop ATPase, both structures contain a structurally conserved P-loop (Fig. 3B), which wraps around the triphosphate through amide-phosphate hydrogen bonds. The ε-amino moiety of the conserved Lys residue (Lys-111 of MvRadA) in the P-loop contacts the γ-phosphate, while the conserved Thr residue (Thr-112 of MvRadA) stabilizes an Mg\(^{2+}\) ion (red sphere, Fig. 3B), which in turn contacts the ATP analog. In the subunit contributing the P-loop, the two putative catalytic residues (Glu-151 and Glu-257 of MvRadA) observed in contact with the candidate for the nucleophilic water (large green sphere, Fig. 3B) are both conserved. Another MvRadA subunit contacts the ATP analog through the ATP cap as well as a portion of the L2 region. Structures of the yeast and E. coli homologs also contain an 8-residue helix starting at the second universally conserved Gly residue (α10, Figs. 1 and 3B) in the L2 region. This helix harbors His-280 of MvRadA, which forms a direct hydrogen bond with the γ-phosphate of AMP-PNP. This residue is conserved as a His in the RadA/Rad51/DMC1 group and as a Phe in the RecA group (Fig. 1). Structural and mutagenic studies on the analogous His-325 of yeast Rad51 suggested the proximity of this residue to ATP (23). Studies on Phe-217 of EcRecA, the analogous residue to His-280 of

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**Table 1**

| X-ray crystallographic data and structure refinement statistics |
|---------------------------------------------------------------|
| X-ray crystallographic data                                   |
| X-ray source                                                 |
| CuK\(_{\alpha}\) radiation, wavelength = 1.5418 Å             |
| Space group                                                  |
| P6                                                            |
| Unit cell dimensions (Å)                                      |
| a = b = 83.9, c = 104.8                                       |
| Resolution range (Å)                                         |
| 40–2.4 (2.49–2.4)                                            |
| Observed reflections                                         |
| 104,732                                                      |
| Unique reflections                                           |
| 14,804                                                       |
| Completeness                                                 |
| 96.0% (94.6%)                                                |
| R\(_{sym}\)                                                  |
| 0.041 (0.29)                                                 |
| I/σ                                                         |
| 11.9 (1.5)                                                   |
| Anomalous Ι/σ                                               |
| 4.1 (2.9)                                                    |
| Crystal structure refinement                                  |
| Reflection with F > 0                                         |
| 13.929/85.2%                                                 |
| R-factor/free R\(_{p}^{2}\)                                   |
| 0.196/0.243                                                 |
| Residues and AMP-PNP                                         |
| 299 and 1                                                    |
| Solvent molecules                                            |
| 106 H\(_{2}\)O, 2 K\(^{+}\) and 2 Mg\(^{2+}\)                |
| Root mean square deviation                                   |
| 0.0066 Å/1.21°                                              |
| bond/angle                                                  |
| Ramachandran                                                 |
| 90.7%                                                        |
| Disallowed                                                   |
| 0                                                            |

\(^{a}\) Values in parentheses refer to values in the highest resolution shell. 
\(^{b}\) R\(_{sym}\) = Σ|h| - (⟨h⟩)/Σ|h|, where (⟨h⟩) is average over symmetry equivalents, and h is reflection index.

> FIG. 3. Filament assembly and ATPase sites. A. filament assembly of 12 subunits. Solvent accessible surface of one central subunit is colored with electrostatic potential (red for negative and blue for positive). The putative ssDNA-binding L1 regions (in green) and L2 regions (in magenta) are located along the filament axis with concentrated positive charge. The ATP analogs are shown in yellow. The NMR-derived dsDNA-binding regions are shown in salmon. B, the ATPase site of MvRadA. C, the ATPase site of molecular chaperone Hsc70 (PDB entry 1HPM). The nucleophilic water, magnesium, and potassium ions are shown as green, red, and purple spheres, respectively.
MvRadA, also suggested an intimate role by this site in ATP binding (39). The structural finding on His-280 of MvRadA and mutagenesis studies on His-325 of yeast Rad51 (23) and Phe-217 of EcRecA (39) imply that the orientation of the 8-residue helix is somewhat similar in RecA-like recombinases. Interestingly, the C terminus of this helix was observed to directly contact both potassium ions (purple spheres, Fig. 3B) in the MvRadA structure. The ATP-stacking Pro-302 in the ATP cap of MvRadA is also conserved (Fig. 1). Despite similarities in their three-dimensional structures, EcRecA apparently does not depend on potassium or other monovalent cations. An interesting question is why EcRecA, and possibly other bacterial homologs as well, does not have such cation dependence. Recently, the hexameric structure of the circadian clock protein KaiC, a known remote RecA homolog, also revealed an ATP-binding site at the subunit interface with a somewhat similar ATP-capping loop in which a lysine-arginine pair (Lys-244/457 and Arg-246/459 in domain I/II) contacts the /H9253-phosphate in trans (40). Similar instances of cationic twins were found in bovine mitochondria F1-ATPase (PDB entry 1BMF) (41) and gene 4 helicase from bacteriophage T7 (PDB entry 1E0J) (42). In the F1-ATPase, one of the cationic residues is provided in trans by the non-catalytic subunit (Arg-373). In the T7 helicase, both cationic residues are provided in trans by the adjacent subunit (Lys-520 and Arg-522). Interestingly, EcRecA has Lys-248 and Lys-250 in its analogous ATP cap (Fig. 1). Both cationic residues were placed in close proximity to ATP in the electron microscopy-reconstructed active EcRecA filaments (25). Such an ATPase site would be unlikely to accommodate monovalent metal ions. The abundance of cationic residues in the ATPase sites of remote RecA homologs also implies a possibility that the cationic side chain of Lys-248, at the equivalent position of Asp-302 of MvRadA, may substitute for one K+ ion, and Lys-250 may substitute for the other. With such a cationic mimicry, RecA is independent of monovalent cation while maintaining ATPase efficiency. Known structures of the three remote RecA homologs appear to suggest that the conserved γ-phosphate-contacting Lys in the P-loop found in ATPases and GTPases (consensus GxxxxGKT/S) (35), though essential, does not suffice to catalyze efficient triphosphate hydrolysis. Coincidently, many small GTPases, which catalyze a similar triphosphate hydrolysis reaction, also require an Arg in trans from GTPase-activating proteins (43).

The structural comparison-implied instance of mimicry between Lys residues and potassium ions may not be alone in nature’s design for ATPases. The ATPase activity of a structurally unrelated molecular chaperone Hsc70 has been observed to be potassium-dependent (44). In the crystal structure of its ATPase domain in complex with ADP and phosphate, two potassium ions have been located by anomalous scattering signals (PDB entry 1HPM) (45). One of the two potassium ions contacts the inorganic phosphate, which occupies a similar position of the γ-phosphate of ATP. The other contacts the β-phosphate of ADP (Fig. 3C). The structure of the ATPase

![Proposed ATPase mechanism.](http://www.jbc.org/)

Glu-151 acts as the general base to activate the hydrolyzing water, which is aligned by hydrogen bonds with Glu-151 and Gln-257. Lys-111, His-280, Mg2+, and two K+ ions polarize the γ-phosphate of ATP. The Mg2+ ions also stabilize the negative charge build-up on the β-phosphate during hydrolysis.
domain of Hsc70 is closely related to actin (46). Interestingly, Lys-18 of actin is located near one K⁺ site observed in Hsc70 (47). In the structures of an actin-mimicking C17K mutant of Hsc70, one potassium ion is indeed displaced by the introduced Lys side chain (47). In the same study, another potassium ion is displaced by the Lys residue which substitutes for Asp-206, a ligand of the displaced potassium ion in the wild-type protein. Both the C17K and D206K mutant proteins are effective in ATP binding but less effective in ATP hydrolysis. Possibly, the catalysis of ATP hydrolysis requires a higher order of precision in the active site. The precision required for optimal ATPase activity can be achieved for both K⁺- and Lys-utilizing enzymes during their respective evolution.

The MvRadA structure determined in the presence of potassium revealed an abundance of four cations (Lys-111, Mg²⁺, and 2 K⁺) and one hydrogen-bond donor (His-280), which contacts the triphosphate of the ATP analog (Fig. 2D). A possible mechanism for the ATP hydrolysis is proposed (Fig. 4). Each oxygen atom of the γ-phosphate interacts with two electron-withdrawing moieties. As such, the γ-phosphate likely becomes highly polarized and susceptible to nucleophilic attack by the hydrolyzing water molecule. The water molecule is held in an optimal location by hydrogen bonds with Glu-151 and Gln-257. Glu-151 is also a candidate for the general base which deprotonates the water. Another possible general base candidate is the γ-phosphate of ATP, which also contacts the hydrolyzing water. The amide groups in the conserved P-loop (not shown in Fig. 4) and the Mg²⁺ ion directly contact the β-phosphate of the leaving ADP. Such proximity to ADP has not been observed for human Rad51 (5) and in 100–200 mM KCl for human DMC1 (47). In the structures of an actin-mimicking C17K mutant of Hsc70, one potassium ion is indeed displaced by the introduced Lys residue which substitutes for Asp-206, a ligand of the displaced potassium ion in the wild-type protein. Both the C17K and D206K mutant proteins are effective in ATP binding but less effective in ATP hydrolysis. Possibly, the catalysis of ATP hydrolysis requires a higher order of precision in the active site. The precision required for optimal ATPase activity can be achieved for both K⁺- and Lys-utilizing enzymes during their respective evolution.

The relationship between the ATPase activity and strand exchange activity of RecA-like recombinases is not clear. The EcRecA K72R mutant (48) and G96D mutant (49), the yeast Rad51 K191R mutant (50), and the human Rad51 K133R mutant (51) were found to be ATPase-inactive but still capable of promoting strand exchange. EcRecA was observed to promote strand exchange in the presence of slowly hydrolyzable ATP analog ATPyS (52) and so was yeast Rad51 (50). But human Rad51 and DMC1 appeared to require ATP for efficient strand exchange (3, 53). Nevertheless, binding of ATP or an ATP analog is required for the hallmark strand exchange reaction. Due to high level of similarity in amino acid sequence, eukaryal Rad51 and DMC1 homologs may share structural resemblance with MvRadA. Sites for binding monovalent cations may also exist in some of these homologs. Interestingly, a study on the effects of divalent cations on human Rad51 suggested that calcium stimulates the strand exchange activity by slowing down the ATPase activity (54). Potassium ions bound at the ATPase site as observed in the MvRadA structure would facilitate the ATP binding and modulate the ATPase activity. As a result, potassium ions may also play a role in modulating the strand exchange activity. Yeast Rad51 was observed to require KCl for many of its functions. The optimal KCl concentration for strand exchange activity was found to be 30–60 mM (4). Optimal strand exchange activities were found in 200 mM KCl for human Rad51 (5) and in 100–200 mM KCl for human DMC1 (3). Ammonium sulfate was also found to stimulate the strand exchange activity of human Rad51 (5, 55). In the presence of NaCl, human Rad51 was found to be ATPase active (34). It is possible that human Rad51 is less selective on monovalent cations. High resolution structures of eukaryal recombinase filaments in complex with known co-factors have not been reported. It remains uncertain whether any of the eukaryal homologs of MvRadA share the monovalent cation-binding sites. Should such sites exist, potassium may be the only physiologically abundant cation to be utilized in modulating the activities of the recombinases.

RecA-like recombinases are fascinating molecules for the study of allosteric regulation. The activation of all known RecA-like recombinases in vivo depends on three co-factors: ssDNA, ATP, and Mg²⁺. Documented experiments in vitro have demon-strated that ssDNA can be substituted by dsDNA, RNA, heparin and high salt and that ATP can be substituted by its analogs resistant to hydrolysis. Two disordered loops L1 and L2 in the crystal structure of EcRecA were initially hypothesized as the binding site for ssDNA (36). These two EcRecA loops are analogous to residues 220–227 and 258–272 of MvRadA. Subsequent efforts on EcRecA were focusing more on the larger L2 loop and its flanking regions. Synthetic peptides (~20 amino acids) in the L2 region of EcRecA were shown to simulate the functions of the intact protein of more than 300 amino acids (56–58), highlighting the importance of the L2 loop. The structure of the yeast Rad51 filament did not show any ordered co-factor (23). A large portion of its L2 region analogous to residues 259–273 of MvRadA was disordered. In the absence of potassium, residues 260–278 of MvRadA was also disordered (24). Incorporation of K⁺ ions into the MvRadA crystal triggered a long range conformational change which orders and stacks the putative ssDNA-binding L1 and L2 regions along the filament axis (green and magenta loops, Fig. 3A), creating a positively charged patch compatible for binding anionic DNA (blue patch, Fig. 3A). It would be difficult to functionally dissect the roles played by the L1 and L2 loops. We did not observe clear sites for Cl⁻ ions. Such sites may be disordered in the structure. Nevertheless, cross-talk between the ATPase site and the ssDNA-binding L2 region of MvRadA has been visualized. The γ-phosphate of the recombinase-bound ATP analog is highlighted by its extensive interactions with the L2 region (Fig. 2D): a direct hydrogen bond with His-280, K⁺-bridged interactions with the helix harboring His-280, and the water nucleophile-bridged hydrogen bond with Gln-257.

Acknowledgment—We thank the Saskatchewan Structural Sciences Centre for access to the X-ray facility.

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Crystal Structure of an ATPase-active Form of Rad51 Homolog from Methanococcus voltae: INSIGHTS INTO POTASSIUM DEPENDENCE
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J. Biol. Chem. 2005, 280:722-728.
doi: 10.1074/jbc.M411093200 originally published online November 10, 2004

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