The Iron-containing Domain Is Essential in Rad3 Helicases for Coupling of ATP Hydrolysis to DNA Translocation and for Targeting the Helicase to the Single-stranded DNA-Double-stranded DNA Junction

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Robert A. Pugh†, Masayoshi Honda‡, Haley Leesley‡, Alvin Thomas†§, Yuyen Lin§, Mark J. Nilges§, Isaac K. O. Cann‡¶**, and Maria Spies‡¶†

From the †Department of Biochemistry, ‡Department of Animal Sciences, and ¶Department of Microbiology, †Illinois EPR Research Center, and **Institute for Genomic Biology, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801

Helicases often achieve functional specificity through utilization of unique structural features incorporated into an otherwise conserved core. The archaeal Rad3 (xeroderma pigmentosum group D protein (XPD)) helicase is a prototypical member of the Rad3 family, distinct from other related (superfamily II) SF2 enzymes because of a unique insertion containing an iron-sulfur (FeS) cluster. This insertion may represent an auxiliary domain responsible for modifying helicase activity or for conferring specificity for selected DNA repair intermediates. The importance of the FeS cluster for the fine-tuning of Rad3-DNA interactions is illustrated by several clinically relevant point mutations in the FeS domain of human Bach1 (FancJ) and XPD helicases that result in distinct disease phenotypes. Here we analyzed the substrate specificity of the Rad3 (XPD) helicase from Ferroplasma acidarmanus (FacRad3) and probed the importance of the FeS cluster for Rad3-DNA interactions. We found that the FeS cluster stabilizes secondary structure of the auxiliary domain important for coupling of single-stranded (ss) DNA-dependent ATP hydrolysis to ssDNA translocation. Additionally, we observed specific quenching of the Cy5 fluorescent dye when the FeS cluster of a bound helicase is positioned in close proximity to a Cy5 fluorophore incorporated into the DNA molecule. Taking advantage of this Cy5 quenching, we developed an equilibrium assay for analysis of the Rad3 interactions with various DNA substrates. We determined that the FeS cluster-containing domain recognizes the ssDNA-double-stranded DNA junction and positions the helicase in an orientation consistent with duplex unwinding. Although it interacts specifically with the junction, the enzyme binds tightly to ssDNA, and the single-stranded regions of the substrate are the major contributors to the energetics of FacRad3-substrate interactions.

The function of many multisubunit DNA repair complexes requires activity of DNA helicases, which are ubiquitous, highly diverse molecular motors that convert the chemical energy of ATP binding and hydrolysis into mechanical work of unidirectional translocation along the DNA lattice (reviewed in Refs. 1, 2). Unidirectional translocation of a helicase may be coupled to other thermodynamically unfavorable processes, including separation of the nucleic acid duplexes and disassembly of protein-nucleic acid complexes. Coupling of ATP hydrolysis to translocation is achieved through the set of so-called “helicase signature motifs” that define the motor core of the enzyme (3, 4). The motor cores of numerous helicases are structurally and mechanistically similar, yet these enzymes display remarkable functional diversity (for review see Ref. 4). It has become clear in recent years that such diversity may be achieved in trans through utilization of specific processivity factors or in cis by incorporating additional structural features that direct interaction with nucleic acids, duplex destabilization, and strand separation activities.

The Rad3 (XPD) helicase from Ferroplasma acidarmanus (FacRad3) provides an example of a helicase that utilizes a unique structural domain. The Rad3 family includes several closely related DNA helicases found in all three domains of life. All members of this family are bona fide DNA helicases that display 5′ → 3′ polarity of ssDNA translocation. These Rad3 family helicases (5) differ from the related SF2 (superfamily II) enzymes because of a unique insertion between signature motifs Ia and II (Fig. 1A) that contains an iron-sulfur (FeS) cluster (6). The presence of an FeS cluster was recently reported for the archaean Rad3 (6) and bacterial DinG (7) helicases and inferred in other related enzymes. However, the mechanistic role of this cluster remains unclear. The FeS cluster-containing insertion may represent an “auxiliary domain” responsible for modifying helicase activity or for conferring specificity for a defined set of DNA structures.

FeS clusters are ubiquitous and ancient prosthetic groups that are essential for fundamental biological processes (reviewed in Ref. 8). The biochemical utility of FeS clusters in contemporary organisms usually rests upon either their ability to accept and donate

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† The on-line version of this article (available at http://www.jbc.org) contains supplemental Methods, additional references, and Figs. S1–S6.

1 To whom correspondence should be addressed: Dept. of Biochemistry, 493 Roger Adams Laboratory/MC-712, University of Illinois at Urbana-Champaign, 600 S. Mathews Ave., Urbana, IL 61801-3602. Tel.: 217-244-9493; Fax: 217-244-5858; E-mail: mspies@life.uiuc.edu.

2 The abbreviations used are: XPD, xeroderma pigmentosum group D protein; dsDNA, double-stranded DNA; DTT, dithiothreitol; FPA, fluorescence polarization anisotropy; FeS, iron-sulfur cluster; NER, nucleotide excision repair; ssDNA, single-stranded DNA; ATP-γS, adenosine 5’-O-(thiotriphosphate); ICP-MS, inductively coupled plasma-mass spectrometry.
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Enzymes and Reagents—Chemicals were of a reagent grade. Restriction enzymes and T4 polynucleotide kinase were from New England Biolabs. Streptavidin was purchased from MP Biomedicals, resuspended in 50 mM Tris-HCl (pH 7.5), 20% glycerol, and 10 mM NaCl, aliquoted, and stored at −80 °C. [γ-32P]ATP was from PerkinElmer Life Sciences. ATP, ATPγS, and poly(dT) were from Sigma. The concentrations of ATP and poly(dT) were measured spectrophotometrically using the extinction coefficients ε264 = 15.4 mM−1 cm−1 (for ATP and ATPγS) and ε264 = 8520 M−1 cm−1 (for poly(dT)).

Cloning and Mutagenesis of the FacRad3 Gene—The rad3 gene (GenBank™ accession number ZP_01709501) from *F. acidarmanus* (25) was amplified by PCR using primers “FacRad3F” and “FacRad3R” introducing the XhoI site at the 3′-end of the termination codon (Table 1). The *F. acidarmanus* genomic DNA, kindly provided by Dr. Jill Banfield (University of California, Berkeley), was used as the template. The PCR product was cloned into pGEMT plasmid, a TA-cloning vector (Promega), sequenced to ensure the integrity of the coding sequence, and subcloned into pET28a plasmid, which allows expression of the protein with the His6 tag at the N terminus. The integrity of the insert was again confirmed through nucleotide sequencing (W. M. Keck Center for Functional and Comparative Genomics, University of Illinois at Urbana-Champaign). The construct was designated pET28/facrad3.

Site-directed Mutagenesis—Each of the four conserved cysteines proposed to be ligands to the putative FeS cluster in FacRad3 helicase were individually substituted with serines. The mutations were carried out using the QuikChange multisite-directed mutagenesis kit (Stratagene) and pET28/facrad3 plasmid as a template, following the procedure described by the manufacturer. The primers used for each mutagenesis reaction...
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**TABLE 1**

| Oligonucleotide | Sequence (5'–3') | Assays |
|-----------------|------------------|--------|
| FacRad3F        | CATATGAAAAATTTACCCCTGGGGGGAATGGCATAGG | FacRad3 cloning |
| FacRad3R        | CTGAGGAAAAATTTACCCCTGGGGGGAATGGCATAGG | Mutagenesis |
| C87S            | GCGAAGCTTATTTTTTTTGGGGAATGGCATAGG | SD/HA/EMSA |
| C105S           | GCGCAGAAAAATTTACCCCTGGGGGGAATGGCATAGG | SD/HA/EMSA |
| C108S           | AATGATTTATTTTTTTTGGGGAATGGCATAGG | SD/HA/EMSA |
| C169S           | GCGAGAATTTTTTTTTTGGGGAATGGCATAGG | SD/HA/EMSA |
| H01             | CCCGATACCAGGGCCGCGC | SD |
| H02             | GCTGAGCGCCTCCTAGCTGGG | SD |
| H0120T          | CCCGATACCAGGGCCGCGC (T)20 | SD |
| H20T02          | (F')GGCAGCTTATTTTTTTTGGGGAATGGCATAGG | SD |
| H20T02Bio       | (F')GGCAGCTTATTTTTTTTGGGGAATGGCATAGG | SD |
| U5T             | (F')GGATTAGTACCTAAGCCATCC | SD |
| U10T            | (F')GGATTAGTACCTAAGCCATCC | SD |
| U15T            | (F')GGATTAGTACCTAAGCCATCC | SD |
| U30T            | (F')GGATTAGTACCTAAGCCATCC | SD |
| U22             | GCAATCCATCTCAGGACCATCC | SD |
| iCy510T         | GATGCTTAGCTAATTTTCATCC/ (T)10 | SD |
| 22Cy5           | GATGCTTAGCTAATTTTCATCC/ (T)10 | SD |
| iAM1            | GATGCTTAGCTAATTTTCATCC/ (T)10 | SD |
| iAM3            | GATGCTTAGCTAATTTTCATCC/ (T)10 | SD |
| iAM7            | GATGCTTAGCTAATTTTCATCC/ (T)10 | SD |
| iAM14           | GATGCTTAGCTAATTTTCATCC/ (T)10 | SD |

*SD indicates streptavidin displacement; HA indicates helicase assay; EMSA indicates electrophoretic mobility shift assay; FQ indicates fluorescence quenching assay; FF indicates fluorescence footprinting; A indicates anisotropy.*

are listed in Table 1. All constructs were confirmed by sequencing. The plasmids expressing mutant FacRad3 enzymes were designated pET28/facrad3-C87S, pET28/facrad3-C105S, pET28/facrad3-C169S, and pET28/facrad3-C108S, respectively.

**Purification of FacRad3 Enzymes**—The His6-tagged wild type FacRad3 helicase as well as FacRad3C87S, FacRad3C105S, and FacRad3C169S, and FacRad3C108S mutants were overexpressed in *E. coli* (BL21-Codon Plus, Stratagene) and purified by the following procedure. *E. coli* cells harboring the plasmid for expression of the wild type or mutant FacRad3 were grown at 37 °C in LB medium supplemented with 100 μg/ml ampicillin until the OD600 reached 0.3. Protein expression was induced by addition of 0.1 mM isopropyl 1-thio-D-galactopyranoside. Induced cells were cultured overnight at 16 °C, harvested by centrifugation, resuspended in lysis buffer (50 mM sodium phosphate (pH 7.4); 500 mM NaCl; 1% v/v Triton X-100; 50 mM imidazole; 5 mM β-mercaptoethanol; Complete mini-EDTA protease inhibitors (Roche Applied Science)), and lysed by sonication. Cell debris was removed by centrifugation, and the clarified lysate was applied to a HiTrap Chelating HP column (GE Healthcare) equilibrated with nickel and stored in buffer Ni-A (50 mM sodium phosphate (pH 7.4); 500 mM NaCl; 300 mM imidazole; 5 mM β-mercaptoethanol). The His6-tagged proteins were eluted in buffer Ni-B (50 mM sodium phosphate (pH 7.4); 100 mM NaCl; 300 mM imidazole; 5 mM β-mercaptoethanol). The Ni-B elution was then immediately applied to a HiTrap Chelating HP column (GE Healthcare) equilibrated with nickel and stored in buffer Ni-B (50 mM sodium phosphate (pH 7.4); 1 M NaCl). Fractions containing FacRad3 were pooled, concentrated, and dialyzed overnight against Storage Buffer (50 mM Tris-HCl (pH 7.5); 200 mM NaCl; 2 mM DTT; 50% glycerol). The purified enzymes were stored at −80 °C. Concentrations of the purified enzymes were determined spectrophotometrically using ε280 = 91300 M⁻¹ cm⁻¹.

**ICP Mass Spectroscopy**—The iron content in the purified proteins was determined by inductively coupled plasma mass spectroscopy. ICP-MS measurements were carried out at the UIUC Microanalysis Laboratory using the SCIEX ELAN DRCE ICP-MS (PerkinElmer Life Sciences).

**Circular Dichroism Spectroscopy**—Circular dichroism spectra were obtained using a Jasco J-720 spectropolarimeter. The far-UV CD spectra (from 200 to 250 nm) were recorded immediately after protein purification using a 1-mm cell in buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 1 mM DTT. Secondary structure content of the wild type FacRad3 and FacRad3C108S proteins was deconvoluted from the experimental data using the DICHROWEB On-line circular dichroism analysis application (26).

**DNA Substrates**—All oligonucleotides used in this study are listed in Table 1. All oligonucleotides were purchased from Operon Biotechnologies, Inc., or Integrated DNA Technologies and were either PAGE- or high pressure liquid chromatography-purified. Where indicated, the oligonucleotides were 5’-radiolabeled using T4 polynucleotide kinase. Excess [γ-32P]ATP was removed by passing the labeled oligonucleotides through Microspin G-25 columns (GE Healthcare) equilibrated in 20 mM Tris-HCl (pH 8.0) and stored at −20 °C. Substrates containing dsDNA regions were produced by annealing the appropriate oligonucleotides in the annealing buffer (5 mM potassium phosphate (pH 6.8), 200 mM KCl, 50 mM Tris-HCl (pH 7.5)). The annealing mixtures were heated to 95 °C for 5 min, and the heat block was then removed and allowed to cool to room temperature for 3 h. The annealed substrates were then buffer-exchanged by passing through Microspin G-25 columns equilibrated in 20 mM Tris-HCl (pH 8.0) and stored at 4 °C.
**Internal Cy5 Labeling of Oligonucleotides for Fluorescence Footprinting Assays**—The iAmMC6T-labeled oligonucleotides were reacted with Cy5 mono-NHS esters (GE Healthcare) according to the manufacturer’s protocol. The length of labeling of amino-modified DNA with Cy5 NHS esters was extended from 1 to 6 h. The labeled oligonucleotides were then purified using MicroSpin G-25 columns followed by ethanol precipitation to remove unincorporated Cy5-NHS esters. The labeling efficiency was determined spectrophotometrically by comparing the ratio of oligonucleotide (measured at 260 nm) and Cy5 dye (measured at 649 nm). Labeling efficiencies for oligonucleotides “iAM1,” “iAM3,” “iAM7,” “iAM14,” and “iAM7Bot” were 125, 119.4, 101.3, 69.6, and 92.6%, respectively (higher than 100% labeling efficiency likely reflects the presence of free dye).

**ATP Hydrolysis Assays**—A Cary 300 Bio spectrophotometer (Varian) was used to monitor ATP hydrolysis coupled to NADH oxidation (27). The reaction buffer contained 20 mM Tris-HCl (pH 7.5), 5 mM MgCl2, 1 mM DTT, 0.2 mg/ml NADH, pyruvate kinase (47 units/ml), lactic dehydrogenase (37 units/ml), 2 mM phosphoenolpyruvate, 10 mM of the indicated enzyme, 10 μM DNA (nucleotides) poly(dT) (ssDNA) or NdeI-linearized pBR322 (dsDNA). Upon preincubation at 37 °C, the reactions were initiated by the addition of ATP (0.01, 0.025, 0.5, 1, or 3 mM, respectively). The rate of hydrolysis was determined spectrophotometrically by comparing the decrease in NADH absorbance measured at 340 nm (s^{-1} × 9820 = the rate of ATP hydrolysis (in μM/min). The steady-state parameters of the ATP hydrolysis were determined by fitting the experimental data to the Michaelis-Menten equation using GraphPad Prism software.

**Streptavidin Displacement Assays**—DNA translocation was observed using streptavidin displacement assays (28). The partial duplex and fork substrates were produced by annealing the “H20T02bio” oligonucleotide to “H01” and “H0120T”, respectively (Table 1). The 32P-labeled oligonucleotide biotinylated at the 3’-end, the 32P-labeled partial duplex or fork substrates biotinylated at the 3’-end of the oligonucleotide on which the FacRad3 helicase is expected to translocate (10 nm of each sub-strate), was incubated with 300 nm streptavidin in reaction buffer containing 20 mM Tris-HCl (pH 7.5), 5 mM MgCl2, 2 mM DTT, 5 mM ATP for 5 min followed by the addition of 6 μM biotin. The zero time points were pulled, and the reactions were initiated by the addition of 50 nm enzyme. Ten μl aliquots were taken at 2- and 5-min intervals and quenched by adding an equal volume of quench solution containing 0.6% SDS, 200 mM EDTA, 10 μM poly(dT) for ssDNA or 10 μM of ssDNA of the same sequence as the radiolabeled strand in the duplex. Then 5 μl of 30% Ficoll 400 (w/v) containing 0.25% bromphenol blue (w/v) and 0.25% xylene cyanol (w/v) was added to the quenched reactions. The reaction products were separated by electrophoresis on a 15% (19:1) native polyacrylamide gel, visualized, and quantified using a Storm 840 PhosphorImager (GE Healthcare) and ImageQuant software. The assays comparing activity of the wild type FacRad3 and FacRad3C1055S enzymes were carried out in the presence of the indicated concentration of each enzyme and were stopped after 2 min.

**Helicase Assays**—The partial duplex DNA with 5’- and 3’-overhangs were produced by annealing the “H20T02” oligo-
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Fluorescent Footprinting Assays—To test how the Cy5 quenching magnitude is affected by the dye position, we annealed a series of forked substrates in which Cy5 dye was incorporated at varying distances from the ssDNA-dsDNA junction (positions −1, +3, +7, and +14). The forked substrates were produced by annealing the Cy5-labeled iAM1 and U15T (“15/20TF−1”), Cy5-labeled iAM3 and U15T (“15/20TF+3”), Cy5-labeled iAM7 and U15T (“15/20TF+7”), and Cy5-labeled iAM14 and U15T (“15/20TF+14”), respectively. The 5′-partial duplex (“15′T+7′”) was the result of annealing the Cy5-labeled iAM7Bot to U15T. Binding assays were carried out as described above under “Fluorescence-based Binding Assays.”

RESULTS

FeS Cluster in FacRad3 Helicases Stabilizes the Elements of Secondary Structure—We have cloned the wild type FacRad3 helicase and its four mutants in which the putative cysteine ligands to the FeS cluster were substituted with serines, the FacRad3C87S, FacRad3C105S, FacRad3C108S, and FacRad3C169S, respectively (Fig. 1A). All five proteins were expressed and purified to homogeneity using identical procedures schematically shown in Fig. 1B. Similar analysis yielded 2.6 iron atoms per FacRad3C105S mutant protein. The three other mutants displayed no iron-specific absorbance around 400 nm indicating the absence of iron in the FeS cluster.

The assays were performed in duplicate.

Fluorescent Footprinting Assays—To test how the Cy5 quenching magnitude is affected by the dye position, we annealed a series of forked substrates in which Cy5 dye was incorporated at varying distances from the ssDNA-dsDNA junction (positions −1, +3, +7, and +14). The forked substrates were produced by annealing the Cy5-labeled iAM1 and U15T (“15/20TF−1”), Cy5-labeled iAM3 and U15T (“15/20TF+3”), Cy5-labeled iAM7 and U15T (“15/20TF+7”), and Cy5-labeled iAM14 and U15T (“15/20TF+14”), respectively. The 5′-partial duplex (“15′T+7′”) was the result of annealing the Cy5-labeled iAM7Bot to U15T. Binding assays were carried out as described above under “Fluorescence-based Binding Assays.”
the FeS cluster in these proteins. Consistently, the ICP-MS analysis of FacRad3C1169S mutant yielded 0.2 mol of iron per mol of protein. Also, similar to SacXPD, EPR analysis of FacRad3 helicase produced spectra consistent with the presence of an FeS cluster (see supplemental Fig. S2).

To determine whether the defect in the FeS cluster affects the structural integrity of purified proteins, we analyzed the far-UV CD spectra for the wild type and mutant proteins (Fig. 1D). The wild type FacRad3 protein and FacRad3C105S mutant produced essentially identical spectra corresponding to a secondary structure content of ~31% α-helices and 20% β-sheets. The amount of secondary structure elements was significantly reduced in the mutants lacking the FeS cluster (Fig. 1D shows a representative spectrum for the FacRad3C1169S mutant protein) indicating that the FeS cluster is important for structural stabilization of the FacRad3 helicase.

The Wild Type FacRad3 and Its Mutants in Which the Cysteine Ligands to the FeS Cluster Are Substituted with Serines Display ssDNA-dependent ATPase Activity—Helicase activity depends on the ability of the enzyme to convert the energy of ATP binding and hydrolysis into ssDNA translocation and duplex destabilization. Using spectrophotometric assays, in which ATP hydrolysis is coupled to NADH oxidation (27), we analyzed ATPase activity of the wild type and mutant FacRad3 enzymes in isolation and in the presence of ssDNA or dsDNA (Table 2). The steady-state rates of ATP hydrolysis by the wild type and mutant FacRad3 helicases were essentially identical and were greatly stimulated in the presence of ssDNA (Table 2). The steady-state rates of ATP hydrolysis by the wild type and mutant FacRad3 helicases were essentially identical and were greatly stimulated in the presence of ssDNA (kcat, for all enzymes was ~10 s-1, kcat(FeS) ≈ 25 μM). In contrast, linear plasmid length dsDNA failed to stimulate ATPase activity.

Integrity of the Iron-containing Domain Is Essential for Coupling ATP Hydrolysis to ssDNA Translocation—Because the helicase activity of S. acidocaldarius Rad3 helicase is sensitive to the integrity of the FeS cluster (6), we analyzed unwinding of several DNA substrates by the wild type and mutant FacRad3 helicases (Fig. 2). As expected for the 5’→3’ helicase, FacRad3 was able to unwind a partial DNA duplex with a 5’-overhang and forked DNA. No unwinding was detected in the case of a blunt duplex or a partial duplex with 3’-overhang. Among mutant enzymes, only the FacRad3C105S displayed helicase activity comparable with that of the wild type enzyme. Notably, this was the only mutant that retained the FeS cluster and the secondary structure of the auxiliary domain. The three mutants lacking the FeS cluster failed to unwind any of the tested DNA substrates.

The observed defect in helicase activity in the mutant FacRad3 enzymes may stem from their inability to translocate along the DNA lattice. Although DNA translocation draws on the ATPase activity of the helicase, the uncoupling of these two processes can be easily overlooked if only the steady-state DNA-dependent ATP hydrolysis is monitored. To directly observe translocation by the wild type and mutant FacRad3 helicases, we carried out streptavidin displacement experiments (28). In these assays, ssDNA translocation of the helicase results in an increased rate of streptavidin dissociation from the oligonucleotide biotinylated at the 3’-end, which can be detected using electrophoretic mobility shift assays (Fig. 3A). As expected, in the presence of ATP, the wild type FacRad3 and FacRad3C105S enzymes facilitated rapid displacement of streptavidin from the 3’-end of the biotin-labeled oligonucleotide (Fig. 3A). In the absence of ATP or in the presence of a nonhydrolyzable ATP analog (not shown), the rate of streptavidin displacement was significantly reduced and was equal to that of the spontaneous dissociation. Under the suboptimal conditions, the wild type FacRad3 and FacRad3C105S helicases displaced comparable amounts of streptavidin (supplemental Fig. S3) indicating that both enzymes actively translocate on ssDNA in the 5’→3’ direction and exert similar forces. In contrast, FacRad3C87S, FacRad3C108S, and FacRad3C169S mutants displayed only a slight ATP-dependent increase in streptavidin dissociation, with a magnitude similar to that observed for the wild type helicase in the absence of ATP (Fig. 3A). This likely represents accelerated dissociation of strept-

| TABLE 2 | The wild type and mutant FacRad3 helicases display similar ssDNA-dependent ATPase activity |
|-----------------|-----------------|-----------------|
| ssDNA (10 μM poly(dT)) | No DNA | dsDNA (10 μM) |
| | kcat | KM | kcat | KM | kcat | KM |
| Wild type FacRad3 | 9.3 ± 0.3 | 23 ± 1 | 0.38 ± 0.02 | 22 ± 6 | 0.6 ± 0.04 | 18 ± 6 |
| FacRad3C87S | 10.8 ± 0.3 | 21 ± 3 | 1.15 ± 0.06 | 45 ± 11 | 1.3 ± 0.1 | 58 ± 9 |
| FacRad3C105S | 10.5 ± 0.4 | 34 ± 5 | 1.1 ± 0.12 | 30 ± 15 | 0.67 ± 0.06 | 28 ± 12 |
| FacRad3C108S | 9.2 ± 0.5 | 20 ± 5 | 1.3 ± 0.1 | 53 ± 24 | 1.4 ± 0.06 | 100 ± 20 |
| FacRad3C169S | 12.5 ± 0.6 | 24 ± 5 | 1.0 ± 0.1 | 36 ± 6 | 1.02 ± 0.01 | 35 ± 2 |

FIGURE 2. Integrity of the iron-sulfur cluster is critical for the helicase activity of FacRad3 enzyme. Quantification of the gel-based helicase assays. Data on the graph represent the average amount of each substrate unwound within 15 min by the FacRad3, FacRad3C87S, FacRad3C105S, FacRad3C108S, and FacRad3C169S enzymes. Error bars represent quantification of the three independent experiments. The inset shows typical example of the helicase assay using the fork substrate.
FIGURE 3. FacRad3 enzyme is an ssDNA translocase, whose translocation depends on the integrity of the auxiliary domain. Streptavidin displacement assays using biotinylated 40-mer ssDNA (A), partial duplex with 20 nucleotides 5’-overhang (B), and fork substrate with two 20 nucleotide-long overhangs (C) decorated with streptavidin at the 3’-end of the strand, on which the enzyme is expected to translocate, were carried out as described under “Experimental Procedures.” The substrates, possible intermediates, and products of the streptavidin displacement reactions are shown on the left of each panel. The enzymes and the times at which the reactions were terminated are indicated in the tables above the respective gels. C, the control lane depicting mobility of the substrate in the absence of streptavidin.

FacRad3 Helicase Does Not Translocate on dsDNA—One possible explanation for preferential unwinding of a fork over a partial duplex substrate suggested that the FacRad3 helicase may specifically recognize and interact with the DNA fork. To probe whether the FeS cluster-containing auxiliary domain plays a role in substrate recognition, we analyzed binding of the FacRad3 helicase to various DNA substrates. First, we carried out the electrophoretic mobility shift experiments (supplemental Fig. S4, A–D). The wild type enzyme and the FacRad3C105S mutants were able to shift ssDNA, partial duplexes with either 3’- or 5’-overhangs, as well as forked substrates. No binding was detected in the case of blunt dsDNA (data not shown). Although somewhat less efficient, DNA binding was also observed in the three mutants lacking the FeS cluster.

Quantitative analysis of the Rad3-DNA interactions was unattainable by the gel-based assays because of rapid dissociation of the enzyme from the complex resulting in a smear on the gels and potentially drastic overestimation of the equilibrium dissociation constants. Therefore, we developed a fluorescence-based equilibrium assay, which allowed us to quantitatively evaluate the FacRad3-DNA interactions. By testing several fluorescently labeled substrates, we found that upon binding to DNA, FacRad3 quenches fluorescence of the Cy5 dye incorporated at the ssDNA-dsDNA junction (Fig. 4A). For all substrates containing the 5’-ssDNA overhang, we observed considerable quenching whose magnitude increased with increasing length of the 5’-overhang and reached a maximum at 15 nucleotides or longer tail. At this length, over 80% of initial Cy5 fluorescence was quenched (Fig. 4B). Among the analyzed mutants, only the FacRad3C105S binding resulted in the quenching signal similar to the wild type enzyme (Fig. 4, B and C) indicating involvement of the FeS cluster in the quenching of Cy5 flu-
varied between 5:1 and 9:1 for the wild-type enzyme (Fig. 4, Table 3). When these fluorescence-based assays were carried out under the stoichiometric binding conditions (low ionic strength and high concentration of the substrate), the magnitude of quenching linearly increased with increasing concentrations of the wild type FacRad3 or FacRad3ClOSS and reached a plateau when ~1 protein was present per each molecule of substrate (Fig. 4, B and C; Table 3) indicating the 1:1 binding stoichiometry. This stoichiometry reflects binding of a helicase to the fork. At saturation, however, more than one helicase molecule may bind to these substrates by virtue of interaction with the ssDNA overhangs. Indeed, interaction between FacRad3 and ssDNA (including the ssDNA regions of the fork and partial duplex substrates) was observed by the electrophoretic motility shift experiments (supplemental Fig. S4) and inferred from the ssDNA-dependent stimulation of the FacRad3 ATPase (Table 2). These interactions, however, are not as specific as interaction with the ssDNA-dsDNA junction and do not contribute to the Cy5 quenching signal.

**The FeS Cluster Containing Domain Targets FacRad3 to the ssDNA-dsDNA Junction**—The three mutants lacking the FeS cluster do not quench Cy5 fluorescence and therefore cannot be tested in the experiments described above. To compare DNA binding of these mutants to that of the wild type FacRad3 and FacRad3ClOSS enzymes, we carried out FPA measurements. To ensure that the observed FPA signal originates from an increase in the volume of the protein-DNA complex and is not the result of the protein binding on the fluorescent dye, we selected the substrate in which the Cy5 dye was incorporated into the dsDNA region 14 bp from the ssDNA-dsDNA junction (Fig. 5A). Binding of neither the wild type enzyme nor of any of the mutants to this substrate resulted in considerable change in the fluorescence of the Cy5 dye. Using this substrate, we carried out the protein titrations under conditions where the wild type enzyme stoichiometrically binds to the forked DNA (supplemental Figs. 5 and S6).

The FPA signal for the three mutants lacking the FeS cluster increased with increasing concentration of the added protein reaching saturation at the anisotropy value of ~0.37 indicating formation of large protein-DNA complexes. The stoichiometry of the FacRad3mut-DNA interactions ranged from 5:1 to 9:1 possibly reflecting a reduced affinity of the mutant proteins for this DNA substrate or that a significant fraction of the mutant enzymes had compromised DNA-binding capacity (Fig. 5A and supplemental Fig. S6A).
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In contrast, the wild type FacRad3 and the FacRad3C105S enzymes displayed biphasic FPA curves (Fig. 5B and supplemental Fig. S6B). Initially, the anisotropy signal increased upon protein addition and reached a plateau at 1:1 complex stoichiometry. Combined with the Cy5 quenching results discussed above (Fig. 4C) these data suggest formation of the 1:1 Rad3-Fork DNA complex in which the protein is positioned at the ssDNA-dsDNA junction. Addition of higher concentrations of the FacRad3 (or FacRad3C105S) protein resulted in continued increase in the FPA signal reflecting binding of additional protein molecules to the ssDNA arms of the fork. Finally, the magnitude of the FPA signal at saturation (−0.32) was lower compared with that observed for the mutants lacking the FeS cluster indicating smaller complexes with less protein molecules bound to each substrate molecule and therefore potentially larger DNA-binding sites in the proteins with an intact FeS cluster. Collectively, the FPA data confirm the presence of a specific high affinity interaction between FacRad3 helicase and the ssDNA-dsDNA junction, which depends on the Rad3-specific auxiliary domain and therefore on the FeS cluster.

FacRad3 Binds Differently to the Fork and Partial Duplex Substrates—To further probe the FacRad3-DNA interactions, we placed the Cy5 label into the duplex part of the fork substrate at 3, 7, and 14 bp from the junction (the substrates are shown in Fig. 6A as 15/20 TF +3, 15/20 TF +7, and 15/20 TF +14, respectively) and into the 3′ overhang 1 nucleotide from the ssDNA-dsDNA junction (15/20 TF −1). Similarly, the “15 T +7” partial duplex substrate contained a Cy5 dye 7 bp from the junction. Because the wild type FacRad3 protein quenches fluorescence of the Cy5 dye, we expected to observe the quenching of the Cy5 fluorophore in positions occluded by the bound protein. When the fork substrates were used in the binding reactions, the magnitude of Cy5 quenching at saturation (50 nM of the wild type FacRad3 protein and 10 nM substrate) was comparable with that measured for the substrates with the dye incorporated directly at the ssDNA-dsDNA junction. Only marginal quenching was observed in the case of the “15/20 TF +7” and “15/20 TF +14” substrates indicating that “ +7” and “+14” positions were not occluded by the bound FacRad3 protein. In contrast, FacRad3 efficiently quenched the Cy5 dye at “+7” position of the partial duplex substrate (“15 T +7”) suggesting a mode of binding different from that of the fork substrate.

ssDNA Is a Major Contributor to the Energetics of Rad3-DNA Interactions—Using the Cy5 quenching assay, we analyzed binding of the wild type FacRad3 helicase and the FacRad3C105S mutant to a set of related DNA substrates containing different lengths of ssDNA (Table 3; Fig. 7, Equation 1, and supplemental Fig. S5).

\[
Y = \Delta F \times \left( \frac{K_d + [DNA]}{X} - \frac{[K_d + [DNA]}{X} \right) \times 4 \times [DNA] \times X \times X
\]

(Eq. 1)

where \(\Delta F\) is the amplitude of the Cy5 quenching at saturation; \(K_d\) is the equilibrium dissociation constant; and [DNA] is concentration of the DNA substrate. As expected, both proteins bound tightly to the fork and partial duplex substrates resulting in quenching of the Cy5 fluorescence.

Analysis of several partial duplexes revealed that the length of ssDNA defines FacRad3 affinity for each of the selected substrates. The 3′ ssDNA overhang contributed to the binding as well. Affinity for the fork substrate with two 10-nucleotide-long ssDNA overhangs was significantly higher than that for the partial duplex with a 10-nucleotide 5′-overhang and comparable with that of the partial duplex with a 15-nucleotide ssDNA tail. In contrast, only the length of the 5′-ssDNA tail defined the magnitude of Cy5 quenching. Thus, although both single- and double-stranded regions of the substrate are involved in the interaction with Rad3 helicase, ssDNA is the main contributor to the energetics of FacRad3-DNA interactions. Importantly, the tighter binding of the FacRad3 helicase to the forked substrates correlates well with...
more efficient unwinding of this substrate by the enzyme. Binding isotherms for the FacRad3C105S mutant were essentially identical to that of the wild type enzyme (supplemental Fig. S5).

The Cy5 quenching-based assay described above provided a convenient way to quantitatively evaluate FacRad3 binding to a variety of substrates and to determine contributions of individual structural features of each substrate to the stability of the protein-DNA complex. Notably, unwinding of the Cy5-labeled fork substrate by the FacRad3 helicase was similar to that of the unmodified DNA (data not shown) indicating that the presence of Cy5 dye does not interfere with FacRad3 activities. This assay will be valuable for analyzing other Rad3 family helicases, including human Bach1/FancJ, XPD, CHL1, and TEL helicases and their mutants, and for establishing how the effect of the disease-causing mutations on the molecular mechanism of these helicases translates into their clinical manifestations.

**DISCUSSION**

A typical helicase includes several discrete mechanistic features as follows: the ATP-dependent nucleic acid translocating motor characterized by the conserved signature motifs; “pin” or “wedge” structural element important for active duplex destabilization; the substrate-recognition and protein-protein interaction domains, as well as domains providing additional catalytic activities (reviewed in Ref. 4). The accessory domains can be found as N- or C-terminal flanking regions or may be inserted within the motor core of the enzyme. DNA helicases of the Rad3 family possess a unique structural element, an auxiliary domain inserted between helicase signature motifs Ia and II (Fig. 1A). The unusual feature of this insertion is the FeS cluster (6). Although the integrity of the cluster is important for DNA unwinding by the archaeal Rad3 enzyme, the mechanism of its involvement in the helicase activity remained unclear.

Here, we demonstrated that folding and stability of the auxiliary domain uniquely characteristic to the Rad3 helicase family requires the presence of the FeS cluster (Fig. 1, C and D). Although all four mutant FacRad3 enzymes in which putative cysteine ligands to the FeS cluster were substituted with serines (FacRad3C105S, FacRad3C105S, FacRad3C108S, and FacRad3C108S, respectively) retained ssDNA-dependent ATPase activity similar to that of the wild type enzyme (Table 2), only the FacRad3C108S was able to translocate on the ssDNA and to unwind DNA duplexes (Figs. 2 and 3). It was also the only mutant enzyme that retained the FeS cluster. These data suggested that the integrity of the auxiliary domain may be critical for coupling the ssDNA-dependent ATP hydrolysis to the translocation by FacRad3 helicase. Alternatively, one may suggest that the impaired streptavidin displacement activity of the mutant enzymes lacking the FeS cluster stems from a DNA-binding defect. However, the observed ssDNA-stimulated ATP hydrolysis combined with the FPA data argue against this proposition.

Our data have also revealed that the fork substrate is preferred by FacRad3 enzyme over the partial overhang. FacRad3 enzyme bound 3-fold tighter to the fork than to the partial duplex with the identical 5′-overhang.

Notably, under conditions where the FacRad3 bound stochiometrically to the partial duplex or fork substrate, we observed only limited helicase activity. At least 10-fold excess of the enzyme was required to catalyze efficient unwinding of the DNA substrates containing 20 bp. This may be an indication of the low processivity of FacRad3 helicase and reflect its role in vivo, and as a component of the TFIIH the FacRad3 helicase is expected to unwind only a short stretch of the DNA duplex. It is also likely that other components of the TFIIH may stimulate the helicase activity of FacRad3 enzyme. Nevertheless, unwinding of the fork substrate was over 5-fold more efficient than unwinding of a partial duplex with the identical 5′-overhang.
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A

Fork

RecA-1

Cy5(+7)

ATP

3'

RecA-2

B

5'-overhang

RecA-1

Cy5(+7)

ATP

3'

RecA-2

FIGURE 8. Role of the putative DNA-binding site located on the auxiliary domain in recognition of the fork substrate by the FacRad3 helicase. A, binding of the FacRad3 protein to the fork DNA results in binding of the two ssDNA arms into two discrete binding sites positioning the enzyme for efficient DNA unwinding. B, when the enzyme interacts with partial duplex DNA, the same ssDNA overhang can be shared between both primary and secondary binding sites. In the case of the partial duplex with the 5'-overhang, this mode of binding may position the dsDNA near the FeS cluster and result in quenching of the Cy5 dye at the +7 position. On the other hand, similar ssDNA binding polarity will result in placing the dsDNA region of the partial duplex with 3'-overhang away from the FeS cluster-containing domain, and therefore no detectable Cy5 quenching.

Because we demonstrated that FacRad3 helicase translocates exclusively on the ssDNA (classifying it as a SF2-B-α enzyme (4)), the preference for the fork substrate does not stem from translocation on the dsDNA substrate without displacing the complementary strand, but rather results from the positioning of the enzyme at the junction in a manner that allows efficient engagement and duplex unwinding. The proximity of the FeS cluster to the ssDNA-dsDNA junction during substrate recognition indicates that the auxiliary domain inserted between helicase signature motifs Ia and II may be directly involved in substrate recognition, possibly through interaction with both arms of the fork.

Besides its role in structural stabilization of the auxiliary domain, the FeS cluster itself may participate in interaction with the ssDNA, possibly through interaction with both arms of the fork over partial duplex substrate can be explained by the presence of an additional ssDNA-binding site on the auxiliary domain. Structurally characterized SF2 helicases (30, 31) bind ssDNA in a site spanning across the interface between two RecA-like folds (Fig. 8, RecA-1 and RecA-2, respectively). Although no structural information is available for SF2 helicases characterized by the 5' → 3' translocation polarity, we expect the Rad3 enzymes to bind ssDNA in a similar site based on the conservation of the helicase signature motifs (Fig. 8). By binding to the ssDNA-dsDNA junction, the FeS cluster-containing domain may act as a wedge separating the two strands of the duplex (Fig. 8A). When presented with a partial overhang, however, the same ssDNA strand may be accommodated by both primary and secondary DNA-binding sites positioning it in an orientation inconsistent with duplex unwinding (Fig. 8B).

The two discrete modes of substrate binding help to explain the discrepancy in quenching of the Cy5 dye upon binding of FacRad3 to 15T +7 (partial duplex) but not to the 15/20TF +7 (fork) substrate as well as the lack of quenching observed upon binding of the enzyme to the partial duplex with 3'-overhang decorated with Cy5 dye at the ssDNA-dsDNA junction (Fig. 8).

Marked preference for the fork substrate by FacRad3 helicase is not unexpected. Similar to its eukaryotic counterparts (Rad3 and XPD helicases), the archaeal Rad3 is expected to participate in the NER and transcription by the RNA polymerase II as a part of the transcription factor TFIIH. TFIIH complex contains two DNA helicases, the 5' → 3' XPD (Rad3) helicase and the 3' → 5' XPB (Rad25) helicase (reviewed in Ref. 32). Helicase activity of human XPD enzyme is crucial for the DNA unwinding at the site of DNA damage repaired by the NER machinery or during promoter opening by TFIIH (33). The substrate recognition as well as initial duplex destabilization can be accomplished in archaeal cells by the partner helicase, XPB (Rad25) (34). Similarly, in human cells, the XPB-p52 complex is required for substrate opening at the damage site but displays dispensable helicase activity (33). Action of the XPB enzyme may provide the fork-like loading site for Rad3 (XPD) helicase. Once loaded, the Rad3 enzyme can unwind a stretch of duplex sufficient for promoter opening or for creating the substrate for the excision step of NER.

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