Mechanism for accurate, protein-assisted DNA annealing by *Deinococcus radiodurans* DdrB

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Accurate pairing of DNA strands is essential for repair of DNA double-strand breaks (DSBs). How cells achieve accurate annealing when large regions of single-strand DNA are unpaired has remained unclear despite many efforts focused on understanding proteins, which mediate this process. Here we report the crystal structure of a single-strand annealing protein [DdrB (DNA damage response B)] in complex with a partially annealed DNA intermediate to 2.2 Å. This structure and supporting biochemical data reveals a mechanism for accurate annealing involving DdrB-mediated proofreading of strand complementarity. DdrB promotes high-fidelity annealing by constraining specific bases from unauthorized association and only releases annealed duplex when bound strands are fully complementary. To our knowledge, this mechanism provides the first understanding for how cells achieve accurate, protein-assisted strand annealing under biological conditions that would otherwise favor misannealing.

Single-strand DNA annealing | DNA repair | *Deinococcus radiodurans* | crystal structure | DdrB

How cells overcome the need to protect ssDNA from forming deleterious secondary structures and at the same time promote accurate strand annealing represents a longstanding and intriguing question in DNA repair. Single-strand DNA-binding proteins (SSBs) safeguard DNA fragments harboring single-strand regions from misannealing at biological temperatures by binding and occluding bases from potential interaction with other strands (1). Overcoming the thermodynamic barrier of accurate annealing under biological conditions requires specialized annealing proteins able to regulate access of unpaired bases from different strands. Although single-strand annealing (SSA) proteins have been identified in organisms ranging from bacteriophage to humans, how they promote faithful annealing of DNA ends containing large single-strand regions has remained unclear.

One of the best examples of single-strand annealing comes from members of the *Deinococcus* family of bacteria, which are renowned for their ability to withstand and accurately repair genome fragmentation, resulting in hundreds of double-strand breaks (DSBs) (2). Up to one-third of these breaks are repaired by a RecA-independent pathway that is dependent on single-strand annealing mediated by DdrB (DNA damage response B) (3–6).

DdrB was first identified from two independent analyses of the IR-induced transcriptional response of *Deinococcus radiodurans* (7, 8). DdrB binds ssDNA (9); however, unlike SSB, DdrB promotes accurate DNA-strand annealing (5, 10), providing evidence for a role in repair by single-strand annealing. This biological role is further supported by fluorescence microscopy data demonstrating recruitment of DdrB to the nucleoid in the early stages of repair (6) and attenuation of RecA-independent single-strand annealing repair in a ΔddrB strain (5). Although computational sequence analysis has delineated three distinct superfamilies of single-strand annealing proteins (11), similarities in function and quaternary structure suggest a shared mechanism despite divergent evolutionary origins (12–14).

Here, we report the structure of a single-strand annealing protein in complex with a partially annealed DNA intermediate to 2.2 Å. In this structure, DNA is bound to a continuous flat surface on one face of a pentameric DdrB ring assembly. Two ring structures come together in a face-to-face arrangement, sandwiching DNA strands at the interface, thereby stabilizing a partially annealed ss/dsDNA intermediate. Importantly, DdrB restricts free access of one-third of bases within the 30 base pair intermediate, thereby preventing misannealing and ensuring sufficient time to further sample full complementarity of bound strands before release of duplex DNA. This model is further validated by biochemical analysis showing that disruption of DdrB–ssDNA interactions, which restrict access of unpaired bases, significantly decreases accuracy of strand annealing. On the basis of these results we present a general mechanism for how single-strand annealing proteins ensure accurate annealing of ssDNA.

**DdrB Promotes Annealing by Sandwiching DNA Strands at the Interface of Two Pentameric Rings**

Prior studies of annealing proteins such as DdrB, ICP8, and Rad52 have led to a general model in which these proteins bind ssDNA along a continuous surface lining one face of their oligomeric ring structures (14–16). To further investigate this surface and its potential role in DNA annealing we determined the crystal structure of DdrB in complex with single-strand DNA. An initial structure with noncomplementary 14b ssDNA strands (Fig. S1) confirmed ssDNA binding along one extended face of a particle. To whom correspondence should be addressed. Email: mjuno@uwo.ca.

**Significance**

During repair of DNA double-strand breaks, cells must accurately anneal broken strands under temperatures that would normally promote mispairing of even small stretches of ssDNA. How single-strand annealing (SSA) proteins such as Rad52 and DdrB (DNA damage response B) overcome this thermodynamic barrier and achieve accurate strand pairing has remained unclear. Our structural studies of DdrB in complex with partially annealed DNA and supporting biochemical data reveal a mechanism for accurate annealing involving DdrB-mediated proofreading of strand complementarity. DdrB promotes high-fidelity annealing by constraining specific bases from unauthorized association and only releases annealed duplex when bound strands are fully complementary. To our knowledge, this work provides the first mechanistic understanding for accurate strand pairing during SSA-dependent DNA double-strand break repair.

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This article is a PNAS Direct Submission. Data deposition: Crystallography, atomic coordinates, and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID code 4NOE).

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DdrB pentameric ring. Unexpectedly, two DdrB ring structures further assembled in a face-to-face complex, effectively sandwiching DNA strands at the interface. In this “encounter complex” each DdrB subunit bound six bases (30 bases per pentamer), with two bases buried and the other four exposed (Fig. S1C). Because ssDNA was not designed for self-complementarity, only partial electron density was observed for DNA at the “sandwich” interface, and most bases were not directly paired to the opposing strand (Fig. S2). Nevertheless, the structure clearly showed where DNA is bound and what an initial encounter complex looks like when noncomplementary strands are present. To further investigate how DdrB promotes accurate annealing we crystallized DdrB bound to ssDNA capable of forming a partially annealed ss/dsDNA hybrid. A substrate consisting of five tandem repeats of 5′-TTGCGC was used, which enables the GCGC motif to in a repeating pattern of two buried through protein interaction (dT1, dT2) and four exposed (dG, dC, dG, dC) (Fig. 2C and Fig. S3). dT1 is held between L87 and L97 (Fig. 3A), whereas dT2 forms a π–π interaction with the phenol group of Y125 (Fig. 3B).

DNA binding to one face of the pentameric ring is consistent with what has been proposed for human Rad52 on the basis of mutational studies (17). In the case of Rad52, a heptameric ring associates with a similar length of ssDNA (28 bases compared with 30 bases in DdrB) (16, 18), with each monomer interacting with four bases (19). Interestingly, Rad52 generates a repetitive pattern of hydroxyl radical protection on bound ssDNA, with three bases protected and the fourth sensitive to hydroxyl attack (19). In light of the current structures it seems likely that Rad52 also binds ssDNA in a repetitious manner.

**Protein–Protein Interaction Between DdrB Rings Mediates DNA Strand Association**

DdrB pentameric rings within the crystal form a face-to-face encounter complex sandwiching DNA strands at the interface (Fig. 2B). Protein–protein interactions between ring structures function to stabilize the encounter complex and also position DNA strands in the correct polarity for proper base pairing. Exposed bases (GCGC motifs) within the encounter complex are held in the correct distance and in the proper orientation to form three hydrogen bonds with each of the corresponding bases from the opposing DdrB pentamer (Fig. 2C). Encounter complex stability is therefore the net result of contributions from weak protein–protein interactions, burying a surface area of 340 Å², and base pairing, which can account for up to an additional 1,150 Å² of surface contact when full-strand complementarity is present. Given the large proportion of stability dependent on base pairing, the degree of strand complementarity appears to function as the main driver of complex stability and longevity. When strand complementarity is absent, an encounter complex could only exist transiently because of the limited amount of protein–protein interaction available. In addition to these contributions to encounter complex stability, DdrB also acts to inhibit nonproductive encounter complexes by limiting the total amount of binding.
DNA-binding residues. (A–C) Magnified views of protein–DNA interactions with nucleic acids dT1, dT2, and dG3–dC6, respectively. Hydrogen bonding and electrostatic interactions are represented by black dashes, and π–π interactions are represented by yellow dashes. Distances are measured in angstroms.

Fig. 3. DNA-binding residues. (A–C) Magnified views of protein–DNA interactions with nucleic acids dT1, dT2, and dG3–dC6, respectively. Hydrogen bonding and electrostatic interactions are represented by black dashes, and π–π interactions are represented by yellow dashes. Distances are measured in angstroms.

energy available through base pairing. By restricting initial base pairing from 30 to 20 nucleotides, DdrB is able to reduce the upper limit of encounter complex stability significantly and therefore ensure that the complex will only remain intact when fully complementary strands are bound. Having more than the necessary number of bases exposed would only serve to make discriminating between full and partial complementarity more challenging. Thus, encounter complex stability mediated by protein–protein, base–base, and protein–base interaction is perfectly balanced to promote strand contact and proper orientation without trapping strands in an unproductive state when non-complementarity is encountered.

Because ssDNA was not preannealed before crystallization, initial interaction of ssDNA with DdrB was not biased toward any particular residues being buried or exposed. Nevertheless, the crystal structure adopted the lowest possible energy state (maximum amount of annealing), implying that ssDNA is able to be repositioned along the DdrB pentamer following initial binding. It will be interesting to determine whether this involves rapid equilibrium between free and bound states and/or sliding of ssDNA strands relative to the encounter complex until optimal base pairing is achieved.

DdrB Promotes Accurate ssDNA Annealing by Restricting Access of Unpaired Bases

Partially restricting access of unpaired bases represents an elegant means of increasing annealing fidelity. If all bases were simultaneously exposed within an encounter complex one would expect there to be limited benefit to annealing accuracy over strands free of secondary structure in solution. Discrimination between accurate and inaccurate annealing would still be confounded by the small differences in binding energies between homo- and hetero-duplex products. Systematically restricting base access would overcome this difficulty by allowing annealing to occur in a controlled two-stage process involving initial sampling for general complementarity with exposed bases (step one) and a second step checking for additional complementarity between buried bases. Staged annealing would also make it possible to sample long stretches of ssDNA (30 base) for complementarity without committing to the release of partially annealed DNA until all bases (including those buried) are verified in a controlled second step.

To determine if DdrB uses a two-stage annealing process that is dependent on initially restricting access to bound bases we compared annealing of DNA strands containing varying degrees of mismatches for WT and mutant DdrB. In this assay, annealing was negligible in the absence of DdrB (approximately 12% ± 3% compared with 58% ± 3% at 30 min) (Fig. 3A). As expected, DdrB significantly stimulated annealing of complementary strands (5) (Fig. 3B); however, at higher protein concentrations (>1 μM) the efficiency of annealing decreased. A second mode of ssDNA binding involving higher-order assembly of DdrB pentamers has been observed (15); however, the functional significance remains unclear. This effect is similar to what has been observed for human Rad52 (20) where reduced annealing at higher protein concentration was attributed to two opposing concentration-dependent modes of DNA binding (wrapped vs. extended) (18). Nevertheless, the fact that DdrB dramatically increases the efficiency of annealing compared with the same strands free in solution underscores the important contribution of protein-mediated interactions within Deinococcus.

Guided by the crystal structure, two mutants altering different aspects of DdrB–DNA interaction were created and tested for their effect on strand annealing. K102A, which disrupts contact with the sugar-phosphate backbone (Fig. 3) and reduces DNA-binding affinity, was included as a negative control (15). Consistent with its DNA-binding defect, K102A displayed no stimulation of DNA annealing (Fig. 4B). A second mutant, Y125A, was designed to permit unrestricted access of one of the two buried bases (dT2). Y125 accounts for most of the direct stabilizing base interaction with tT2 (Fig. 3B). When substituted to an alanine, this loss of base interaction resulted in a significant enhancement in annealing activity relative to WT protein (Fig. 4B), suggesting the release of buried bases within the encounter complex increased binding energy of the initial encounter complex. To further test effects of Y125A on annealing fidelity, two additional ssDNA oligos with decreasing complementarity were analyzed. Substrates 12x (one mismatch site every 12 bases) and 6x (one mismatch site every 6 bases) were assessed by the same annealing assay with both WT DdrB and Y125A. Although Y125A was as efficient in annealing mismatched substrates as perfectly complementary strands (Fig. 4C), WT DdrB displayed decreasing annealing efficiency with increasing numbers of mismatches and surprisingly did not significantly improve annealing fidelity relative to the unassisted control. Under more biological conditions where other factors such as SSB are present, DdrB may have increased annealing fidelity. Nevertheless, the remarkable effect of Y125A clearly demonstrates the critical importance of restricting bases at the encounter complex stage to ensure optimal protein-assisted annealing fidelity.

A Mechanism for DdrB-Mediated Annealing

Results presented here provide structural evidence for a mechanism of protein-mediated single-strand DNA annealing, which we term “Restricted Access Two-Step” ssDNA annealing (RATS). The structure of DdrB–ssDNA confirms earlier predictions that single-strand annealing proteins such as DdrB and Rad52 are able to bind ssDNA in an extended state along one
continuous surface of a protein oligomeric ring that ensures strands are presented free of secondary structure in a high-energy state. Unexpectedly, the interaction does not permit equal access of all bases to solvent, but rather systematically buries two out of six bases within each subunit (Fig. 2). This mode of ssDNA binding is not dependent on DNA sequence or degree of strand complementarity, as a structure with entirely altered sequence features with other DNA and RNA single-strand annealing proteins despite these proteins not being true homologs. Of note, Rad52 (16, 17), Redβ (12), Erf (22), RecT (23), Sak (24), ICP8 (14), and Hfq (25) assemble into multisubunit ring structures similar to DdrB. Conservation of ring structures among single-strand annealing proteins, despite a lack of sequence or structural similarity, is likely the result of convergent evolution. Multisubunit ring structures represent a simple way of forming discretely sized (20–30 bases) nucleic acid-binding interfaces that permit association of nucleic acid strands in a high-energy state free of secondary structure.

A Mechanism for Single-Strand Annealing

The structure of the DdrB–ssDNA complex shares several key features with other DNA and RNA single-strand annealing proteins despite these proteins not being true homologs. Of note, Rad52 (16, 17), Redβ (12), Erf (22), RecT (23), Sak (24), ICP8 (14), and Hfq (25) assemble into multisubunit ring structures similar to DdrB. Conservation of ring structures among single-strand annealing proteins, despite a lack of sequence or structural similarity, is likely the result of convergent evolution. Multisubunit ring structures represent a simple way of forming discretely sized (20–30 bases) nucleic acid-binding interfaces that permit association of nucleic acid strands in a high-energy state free of secondary structure. Another ssDNA-binding protein, ICP8 (14), is thought to form face-to-face arrangements of stacked ring structures in which DNA strands are presented at the interface. In the case of ICP8, an electron microscopy reconstruction clearly illustrates a face-to-face orientation of oligomeric rings that is ssDNA-dependent; however, because of limited resolution, the EM structure could not inform definitively on the position of DNA or the detailed mechanism of strand annealing (14). The structure of Rad52 is not compatible with a stacked face-to-face complex and may involve a distinct mechanism from DdrB for strand annealing. However, without an ssDNA-bound Rad52 structure, it is difficult to say whether some rearrangement of the protein would not occur, promoting a face-to-face complex. Similar questions remain for how small noncoding regulatory RNAs are annealed with transencoded
target mRNAs by the ring structure of Hfq. Hfq, a well-characterized member of the Hfq–Sm–LSm protein family (26), has been crystallized in complex with ssRNA (25). In this structure, ssRNA is bound along a continuous surface of a hexameric Hfq ring, with each monomer positioning two bases inward and one outward. Although the precise mechanisms are likely to differ on the basis of biological function, it will be interesting to see whether it is possible to trap an Hfq encounter complex with

Fig. 5. DdrB-mediated ssDNA annealing. A face-to-face arrangement of DdrB nucleoprotein complexes facilitates the search for homology between two strands of ssDNA. (A) Base-pairing interactions between exposed bases transiently stabilize the complex, and the buried bases (associated with L87, L97, and Y125) sample the opposing strand for complementarity. (B) Accurate pairing of buried bases, as modeled, results in release of the bound DNA (C). (D) Mispairing of buried bases, as modeled, results in a continuation of the search for homology or dissociation of the decameric complex with DNA still bound to DdrB (E).
partially annealed RNA duplex and whether annealing proceeds through a staged annealing mechanism similar to DNA annealing.

The structure of encounter complexes bound to noncomplementary and cDNA strands presented here provides high-resolution insight into a mechanism of ssDNA annealing. Findings are consistent with previous observations from other SSA proteins, such as ICPS, and reveal how cells can use a multistep annealing mechanism involving restricted access of bound bases to overcome the difficult challenge of protecting single-strand DNA from degradation and secondary structure formation and at the same time promote single-strand annealing.

Experimental Procedures

**Crystallization.** DdrB1–146 from *D. radiodurans* was expressed and purified as reported previously (21). Crystals were grown by the hanging-drop vapor diffusion method. Briefly, 1 μL of DdrB1–146 ssDNA (480 μM protein, 143 μM DNA) was mixed with 1 μL of crystallization condition (6% [wt/vol] PEG 2000, 0.1 M Hepes pH 6.5, 0.05 M CaCl2) and 0.2 μL of Hampton Additive Screen condition 48 (0.01 M l-Glutathione reduced, 0.01 M l-Glutathione oxidized) and equilibrated over 1.2 M ammonium sulfate at 20 °C. No additional cryo-protector was required for data collection. Diffraction data were collected at a wavelength of 1.1 Å on beamline x25 at the National Synchrotron Light Source at Brookhaven National Laboratory. Decameric DdrB–ssDNA complex formed before crystallization.

**Structure Solution.** The diffraction data were integrated with mosfim (27) and then scaled, merged, and converted to structure factors with CCP4 (28, 29). Structure solution was performed with AutoMR from the Phenix software package (30) using the DdrB1–146 monomer from *D. radiodurans* (PDDB 4HQ8) as a search model. The best molecular replacement solution was generated using a search model with manually truncated loops regions, which was then rebuilt-in-place into a simulated annealing OMIT map using Phenix-AutoBuild. Missing loop regions (87–101 and 125–128) and DNA bases were placed manually and refined in iterative cycles using Coot (31) and Phenix-Refine until R and Rfree values converged and geometry statistics reached suitable ranges (Table S1).

Validation of the final structure using Ramachandran analysis indicated that 99.1% of amino acids fell within favorable regions (0% outliers).

**DNA Substrates.** DNA substrates used in crystallization and annealing assays were purchased from Integrated DNA Technologies. The substrate used to generate cryocrystals containing a ss/dsDNA hybrid consisted of a single oligonucleotide (5′–TTGGCTGCTTGGTGCCTCTGGCCGTCCTGG–3′). The 6FAM labeled 60-base probe (5′–ACACTAGTGGTTGCTACACGATACACTAACAGCCTGTAGCATTCCACAGACACGCCCGC–3′) is complementary to a region of M13mp18, whereas substrates 12x (5′–ACACTAGTGGTTGCTACACGATACACTAACAGCCTGTAGCATTCCACAGACACGCCCGC–3′) and 6x (5′–ACACTAGTGGTTGCTACACGATACACTAACAGCCTGTAGCATTCCACAGACACGCCCGC–3′) are complementary to the same region with one mismatch every 12 or 6 bases, respectively.

**Annealing Assay.** DdrB mutants (K102A and Y125A) were generated by site-directed mutagenesis of the WT expression vector (pPROEX-HT DR0070) and were purified as previously described (15). Single-strand DNA annealing activity of DdrB was assessed using a 60-base 5′–6FAM labeled probe (5 nM) complementary to a region of M13mp18 (1 nM). Annealing reactions (25 μL) were carried out at 32 °C in annealing buffer [50 mM Tris pH 7.5, 100 mM NaCl, 25 mM Mg(CH3COO)2, 1 mM DTT, 5% (vol/vol) glycerol] and were initiated by the addition of the 60-base probe (1.25 μL of 100 nM). Reactions were quenched with 2.5 μL of 2.5 μL unlabeled oligomer in 5% (vol/vol) SDS and resolved using a 1% agarose gel run in TBE buffer (90 mM Tris-borate pH 8.3, 2 mM ethylenediaminetetraacetic acid) at 50 V. Annealing reaction products were quantified with a GE Amersham Typhoon Trio+ variable-mode imager and ImageJ (32) and were assessed relative to a controlled annealing reaction carried out in a thermocycler in the absence of protein.

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