The *Thermus thermophilus* DEAD box helicase Hera contains a modified RNA recognition motif domain loosely connected to the helicase core

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ABSTRACT

DEAD box family helicases consist of a helicase core that is formed by two flexibly linked RecA-like domains. The helicase activity can be regulated by N- or C-terminal extensions flanking the core. *Thermus thermophilus* heat resistant RNA-dependent ATPase (Hera) is the first DEAD box helicase that forms a dimer using a unique dimerization domain. In addition to the dimerization domain, Hera contains a C-terminal RNA binding domain (RBD) that shares sequence homology only to uncharacterized proteins of the *Deinococcus/Thermus* group. The crystal structure of Hera_RBD reveals the fold of an altered RNA recognition motif (RRM) with limited structural homology to the RBD of the DEAD box helicase YxiN from *Bacillus subtilis*. Comparison with RRM/RNA complexes shows that a RNA binding mode different than that suggested for YxiN, but similar to U1A, can be inferred for Hera. The orientation of the RBD relative to the helicase core was defined in a second crystal structure of a Hera fragment including the C-terminal RecA domain, the dimerization domain, and the RBD. The structures allow construction of a model for the entire Hera helicase dimer. A likely binding surface for large RNA substrates that spans both RecA-like domains and the RBD is identified.

Keywords: RNA helicase; DEAD box protein; Hera; ATPase; RNA recognition motif

INTRODUCTION

RNA helicases of the DEAD box family couple ATP hydrolysis to rearrangements of RNA structures (Hilbert et al. 2009) and are involved in many facets of RNA metabolism. They share a core domain that is subdivided into two RecA-like domains (RecA_N and RecA_C) that contain all conserved helicase motifs (Gorbalenya and Koonin 1993) involved in nucleotide binding, RNA bind-

ing, and coupling of ATP hydrolysis to RNA unwinding. A flexible linker allows for very different juxtapositions of the RecA-like domains. RNA helicases undergo large conformational changes during their catalytic cycle (Theissen et al. 2008), and an array of open helicase conformations has been structurally characterized (Caruthers et al. 2000; Story et al. 2001; Cheng et al. 2005; Andersen et al. 2006). In a few instances, such as eIF4A-III in the exon junction complex (Andersen et al. 2006; Bono et al. 2006), the *Drosophila* Vasa RNA helicase (Sengoku et al. 2006), and Dbp5/Ddx19 (Collins et al. 2009; von Moeller et al. 2009), closed conformations were obtained in complex with short oligo-U nucleotides and a non-hydrolyzable ATP analog. DEAD box proteins in general display little sequence specificity for RNA. For a few representatives, structures formed by specific RNA sequences are specifically recognized by C-terminal RNA binding domains (RBD). Examples are the *Escherichia coli* RNA helicase DbpA and its homolog from *Bacillus subtilis* (YxiN), as well as *Thermus thermophilus* Hera. All proteins bind and unwind a helix adjacent to a stem-loop structure in 23S rRNA (Diges and

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Abbreviations: RBD, RNA binding domain; RRM, RNA recognition motif; Hera, heat-resistant RNA-dependent ATPase; RMSD: root-mean-square deviation; RecA_N, N-terminal RecA-like domain; RecA_C, C-terminal RecA-like domain; Hera_RBD, Hera residues 424–510; Hera_208–510, Hera including the second RecA-like domain, the dimerization motif, and Hera_RBD.

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Uhlenbeck 2001; Kossen et al. 2002; Linden et al. 2008). Instead of RNA specificity, additional domains flanking the helicase core may mediate nonspecific binding of RNA with high affinity. Examples are the splicing DEAD box proteins Mss116 from yeast and Cyt-19 from *Neurospora crassa* that bind structured RNA with high affinity via basic C-terminal domains and act as general RNA chaperones (Huang et al. 2005; Mohr et al. 2006; Tijerina et al. 2006; Grohman et al. 2007; Halls et al. 2007; Mohr et al. 2008).

The structure of the C-terminal RBD of the RNA helicase YxiN has been determined (Wang et al. 2006) and reveals an RNA recognition motif (RRM) comprising a central β -sheet, flanked by two α -helices on one side. Mutagenesis studies suggest that RNA binding does not involve conserved aromatic residues on the exposed surface of the β -sheet, and indicate a noncanonical mode of RNA binding. Overall, the mode of RNA recognition by DEAD box protein RBDs is currently not understood, and a model for binding of large RNA substrates to RNA helicases and the structural basis of RNA sequence specificity is lacking. Furthermore, the positioning of RBDs in the context of the helicase remains elusive.

We determined two crystal structures of C-terminal fragments of Hera from *T. thermophilus* (termed Hera_RBD and Hera_208-510). Hera_RBD adopts the fold of a degenerated RRM. A linker sequence connects Hera_RBD to the dimerization domain and places it on top of the C-terminal RecA-like domain. Assembly of the entire Hera helicase dimer via structure-based alignment with RNA complexes of other RNA helicases results in a model that allows definition of a likely RNA binding surface extending from the helicase core to the RBD.

RESULTS

Full-length Hera from *T. thermophilus* strain HB27 is a DEAD box helicase (Fig. 1A) that binds fragments of 23S rRNA and RNase P RNA via its C-terminal domain (Linden et al. 2008). The structure of the N-terminal RecA-like domain in complex with AMP has previously been described (Rudolph et al. 2006). Recently, we determined the structures of Hera fragments encompassing the C-terminal RecA-like domain, and established Hera as a stable dimer both in crystals and in solution (Klostermeier and Rudolph 2009). These structures revealed the approximate location of an additional, almost spherical domain, the Hera RBD. The high-resolution structure of the RBD and its orientation relative to the helicase core, however, are currently unknown. We report here the structures of Hera_RBD and of Hera_208-510, which contains the C-terminal RecA-like domain, the dimerization domain, and Hera_RBD. The structures were refined to resolutions of 1.8 Å and 2.8 Å, respectively. Data collection and refinement statistics are summarized in Table 1.

FIGURE 1. Hera_RBD structure sequence relationship and architecture. (A) Domain organization of Hera. The boxes match the domain sizes and the boundaries are indicated. Hera_RBD is located at the C terminus of Hera. The dimerization domain (DD) connects the RBD to the helicase core, which consists of two RecA-like domains termed RecA_N and RecA_C. (B) Sequence alignment of the *T. thermophilus* Hera RBD with putative helicases from *T. aquaticus*, *D. geothermalis*, *D. radiodurans*, *D. deserti*, and structure-based alignment with *B. subtilis* RNA helicase YxiN. The C-terminal sequences of the *T. aquaticus* and *Deinococcus* proteins have been truncated. The secondary structure elements of Hera_RBD are indicated on the top while those of the *B. subtilis* RBD are shown at the bottom for comparison. The sequence numbering corresponds to the full-length Hera. Blue and green arrows denote the sequence ranges that are visible in the Hera_RBD and YxiN structures. (C) Stereo view of the small globular N-terminal domain of Hera_RBD forming a hydrogen bond network with residues from $\beta 1$ and $\beta 3$. The experimental SIRAS electron density map is shown for Trp435 and Tyr472 at 2σ . The backbone trace of the small globular N-terminal domain is shown in yellow. The side-chains are depicted as stick models colored according to atom type. (D) Ribbon diagram of Hera_RBD with helix $\alpha 1$ in red and the β -strands in blue. The view is rotated 180° about the x-axis compared to C. (E–G) Comparison of Hera_RBD with other RRM structures. Hera_RBD is colored as in C. The α -helices and β -strands of the compared structures are colored green and cyan, respectively. (E) The best DALI hit is the human negative elongation factor E (Rao et al. 2006). (F) The closest structural homolog from a prokaryote is BT0572, a protein of unknown function from *B. thetaiotaomicron*. (G) The structurally closest module from a RNA helicase is *B. subtilis* YxiN. The discontinuities in the YxiN structure are marked as spheres connected by dashed lines. Note the absence of the second α -helix in Hera_RBD.

TABLE 1. Data collection, phasing, and refinement statistics

Data set	3I31-RBD native	RBD iodide	3I32-Hera_424-510
Data collection	45.6–1.8	45.8–2.14	44.0–2.8
Resolution range (Å) ^a	(1.84–1.80)	(2.19–2.14)	(2.87–2.80)
100% criterion (Å) ^b	1.80	2.14	2.80
Space group	P4 ₁ 2 ₁ 2	P4 ₁ 2 ₁ 2	P4 ₁ 2 ₁ 2
Cell dimensions (Å)	a = 37.5, c = 136.9	a = 37.4, c = 137.4	a = 65.0, c = 153.6
Unique reflections	9812 (457)	5942 (279)	8706 (513)
Multiplicity	5.4 (5.5)	24.7 (14.8)	49.1 (52.5)
Completeness (%)	99.8 (100)	99.2 (89.1)	99.9 (100)
R _{sym} (%) ^c	7.5 (70.6)	13.9 (73.0)	7.4 (57.6)
Average I/σ(I)	12.2 (1.6)	26.1 (2.6)	37.7 (7.5)
Phasing			
Resolution range (Å)	—	20–2.5	—
CC All/weak ^d	—	32.3/23.3	—
FOM ^e	—	0.38/0.55	—
Phase error (°) ^f	—	36.7	—
Refinement	34.0–1.8	—	39.4–2.8
Resolution range (Å)	(2.06–1.80)	—	(3.0–2.8)
R _{cryst} (%) ^g	20.0 (22.4)	—	25.3 (32.8)
R _{free} (%) ^g	22.4 (27.3)	—	29.3 (35.9)
Number of residues/waters	76/39	—	276/0
Phase error (°) ^h	23.0	—	32.6
RMSD bonds/angles (Å, °)	0.008/1.20	—	0.008/1.16
Ramachandran plot (%) ⁱ	94.5/5.1/0	—	88.7/10.9/0.4

^aValues in parenthesis correspond to the highest resolution shell.^bThe 100% criterion was calculated using SFTOOLS and represents the resolution of a 100% complete hypothetical data set with the same number of reflections as the measured data.^c $R_{\text{sym}} = 100 \cdot \sum_h \sum_i |I_i(h) - \langle I(h) \rangle| / \sum_h \sum_i I_i(h)$, where $I_i(h)$ is the i^{th} measurement of reflection h and $\langle I(h) \rangle$ is the average value of the reflection intensity.^dCorrelation coefficient from SHELXD.^eFigure of merit for phasing using data to 2.5 Å (SOLVE) and 1.8 Å (RESOLVE) resolution.^fCalculated using PHISTATS as comparison to σ_A -weighted model phases.^g $R_{\text{cryst}} = \sum ||F_o| - |F_c|| / \sum |F_o|$, where F_o and F_c are the structure factor amplitudes from the data and the model, respectively. R_{free} is R_{cryst} with 5% of test set structure factors.^hBased on maximum likelihood.ⁱCalculated using PROCHECK. Numbers reflect the percentage amino acid residues of the core, allowed, and additional allowed regions, respectively.

Overall structure of Hera_RBD

The Hera RBD is responsible for binding of Hera to hairpin 92 of 23S rRNA (Supplemental Fig. 1). A BLAST search did not reveal any structurally characterized protein with significant sequence homology for Hera_RBD. The structure of the C-terminal RBD of the *B. subtilis* RNA helicase YxiN was determined and revealed a classical RRM fold (Wang et al. 2006). However, the Hera and YxiN RBDs do not share significant homology (Fig. 1B), and molecular replacement attempts using the *B. subtilis* structure were unsuccessful. The structure of Hera_RBD was therefore determined by SIRAS phasing using a KI-derivatized crystal (Fig. 1C; see Materials and Methods). Residues 424–498 are well ordered in the experimental electron density. The C-terminal sequence 499–EIPSRTRRPARA-510 is disordered in the crystals.

Despite the lack of sequence homology with the YxiN RBD, the structure of Hera_RBD reveals that residues 434–491 fold into a compact, RRM-like domain that is flanked by ordered structures at the termini (Fig. 1D). RRMs are

predominant in eukaryotes, where they function as protein–protein or RNA recognition modules (Clery et al. 2008). RRMs usually display a $\beta 1$ – $\alpha 1$ – $\beta 2$ – $\beta 3$ – $\alpha 2$ – $\beta 4$ topology of secondary structure elements that form a central, antiparallel β -sheet with the α -helices packed against one side of the sheet, leaving the opposite side open for RNA contact. Classical RRMs share a RNP1 sequence ($\beta 3$) with the consensus [RK]-G-[FY]-[GA]-[FY]-[ILV]-X-[FY], and a RNP2 sequence ($\beta 1$) with the consensus [ILV]-[FY]-[ILV]-X-N-L. The corresponding sequences in Hera_RBD, 468-E-G-G-F-Y-V-D-L-475 (RNP1) and 438-Y-K-A-T-G-P-443 (RNP2), show major deviations from this consensus. Furthermore, the classical RRM fold is broken: The $\alpha 2$ -helix is replaced by an extended, well-defined structure connecting $\beta 3$ and $\beta 4$. The reason for this deviation is apparent from the sequence of Hera (Fig. 1B). In the region of the $\alpha 2$ -helix Hera_RBD lacks eight residues compared to YxiN, preventing the formation of a helix that could span the distance between the $\beta 3$ and $\beta 4$ strands. Other Hera homologs from *Thermus aquaticus*, *Deinococcus geothermalis*,

Deinococcus radiodurans, and *Deinococcus deserti* (Fig. 1B) display a similarly shortened sequence as Hera. These bacteria belong to the *Deinococcus/Thermus* (D/T) group and are the only group in which a helicase with sequence homology with Hera has been identified (Linden et al. 2008). Most likely, these helicases have the same topological features in their C-terminal domains and share similar functions.

A search for structural homologs to Hera_RBD in the DALI database (<http://www2.ebi.ac.uk/dali>) returned 165 low significance hits with Z-scores between 3.5 and 6.1. Scores <2.5 are defined as structurally dissimilar and the self-score of a domain of equivalent size is ~20. Many of the identified proteins are involved in RNA binding and/or processing, and 2/3 of all structures with DALI scores >4.5 are eukaryotic RRM. The closest structural homolog is the human negative elongation factor E (Z-score 6.1; RMSD 2.7 Å over 64 residues) that may be involved in transcriptional regulation of HIV-I (Fig. 1E; Rao et al. 2006). The closest prokaryotic homolog is BT0572 (Fig. 1F), a protein of unknown function from *Bacteroides thetaiotaomicron* (Z-score 5.2; RMSD 2.8 Å over 62 residues; unpublished PDB-ID 2f06). The best match to a bacterial RNA helicase RRM is that of *B. subtilis* YxiN (Z-score of 4.5; RMSD 2.3 Å over 51 residues) (Fig. 1G; Wang et al. 2006). The prime differences between Hera_RBD and the structures identified in the DALI search are the absence of helix $\alpha 2$ in Hera, but also the length of helix $\alpha 1$ and its angular disposition to the β -sheet. Of note, the ACT domain in *T. thermophilus* aspartokinase (Z-score 4.5; RMSD 3.4 Å over 61 residues) shows comparable structural homology with Hera_RBD as the RRM domains discussed above. ACT domains are small molecule or protein binding entities in cytosolic proteins involved in metabolism, transport, and transcriptional regulation (Grant 2006). While ACT domains share the same topology as RRM, they are not involved in RNA binding. Taken together, Hera_RBD shares structural similarity with the RRM and ACT domains but differs significantly not only on the sequence, but also on the secondary structure level.

The RRM in Hera_RBD is flanked by sequences that are neither part of the dimerization domain nor flexible linkers between domains but adopt well-defined structures. The N-terminal 12 residues 424-AERSLLTGEEGW-435 fold into a small globular structure containing two β -turns that attach to the central β -sheet of the RRM (Figs. 1C, 2A). Seven out of these 12 residues are identical in the D/T group RNA helicases (noted in boldface). Hydrogen bonds are formed between the side-chains of Ser427 and Asp474, and between the amide nitrogen of Leu428 and Thr437. Asp474 and Thr437 of the RRM are also conserved, underscoring the importance of these interactions for D/T group helicases. In addition, the conserved leucine residues 428 and 429 are engaged in hydrophobic interactions with the β -sheet and the sequence C-terminal to the RRM. In Hera₂₀₈₋₅₁₀, the small N-terminal motif mediates the contact of Hera_RBD with the C-terminal RecA domain (see below). Residues

FIGURE 2. Comparison of Hera with other RRM domains and attempt to delineate the RNA binding mode. (A) Hera_RBD, (B) YxiN. (C) U1A with RNA bound across the β -sheet. (D) SRp20 with RNA bound at the $\beta 2/\beta 3$ loop. Residues on the β -sheet that bind RNA are drawn as stick models. Equivalent residues are drawn for Hera and YxiN for which the RNA binding modes are unknown. (E) Placement of the U1A-bound RNA from C into a cleft of Hera_RBD. Hera_RBD is colored as in Figure 1D and the surface is rendered transparent. The U1A-RNA is shown as a stick model with carbon atoms colored green. (F) View along the principal axis of the RNA.

492-RRVEGLLEIPSRTRRP-510 follow the Hera_RBD. Crystal contacts and interactions with the N-terminal motif noted above impose an extended conformation for the first seven residues (underscored) (Fig. 1D), while the rest are disordered. Several positively charged residues in this region

may hint to unspecific RNA binding, but the sequence conservation among the D/T group helicases is low.

Comparison with YxiN and analysis of potential RNA binding surfaces in Hera_RBD

Although there is no significant sequence homology between the two RBDs (Fig. 1B), a functional homology of YxiN and Hera in 23S rRNA binding is likely. The $\beta 1/\alpha 1$ and $\alpha 2/\beta 4$ loop regions in YxiN contain various lysine residues and have been suggested to be involved in RNA binding (Wang et al. 2006). While the loops are disordered in YxiN and may only become ordered upon RNA binding, these loops have well-defined conformations in Hera_RBD (Fig. 2A,B). However, in Hera_RBD only a single positively charged residue, Arg444, is present in $\beta 1/\alpha 1$ and no positive charges are present in $\alpha 2/\beta 4$, rendering it unlikely for these loops to contribute to RNA binding to the same extent as suggested for YxiN. Thus, the RNA binding modes of Hera and YxiN may be very different.

RRMs in general utilize a variety of RNA binding modes (Maris et al. 2005; Dominguez and Allain 2006; Clery et al. 2008). The classical mode of RNA binding involves tyrosine or phenylalanine residues in $\beta 1$ and $\beta 3$ on the solvent-accessible side of the β -sheet. The aromatic side-chains stack onto nucleobases and the RNA is bound diagonally across the β -sheet (Clery et al. 2008). This binding mode is exemplified in the complex of the splicesosomal U1A protein with RNA (Fig. 2C; Oubridge et al. 1994) or in complexes of human UP1 with several oligonucleotides (Myers and Shamoo 2004). A distinct RNA binding mode is present in the pre-mRNA splicing factor SRp20 (Hargous et al. 2006). SRp20, similar to the classical binding mode, binds RNA bases at the two aromatic residues in $\beta 1$ and $\beta 3$, but in addition contacts the RNA via a long $\beta 2/\beta 3$ loop (Fig. 2D). As a consequence, the RNA is not bound diagonally across the β -sheet, but perpendicular to the strands at the lower half of the β -sheet. Furthermore, so-called quasi-RRMs have been described that presumably bind RNA exclusively via loops $\beta 1/\alpha 1$ and $\beta 2/\beta 3$, without contacts to residues of the β -sheet (Dominguez and Allain 2006). Quasi-RRMs contain a characteristic β -hairpin inserted in $\alpha 2/\beta 4$ that may partake in RNA binding, but the structure of a quasi-RRM/RNA complex is currently unknown. Intermediate RNA binding modes to the ones described above exist, pointing to a whole spectrum of RNA-RRM positioning.

In order to define the probable RNA binding site on Hera_RBD, we compared the poses of RNA molecules in known RRM/RNA complexes with the surface properties of Hera_RBD. Superposition of the SRp20/RNA complex with Hera_RBD shows severe clashes of the RNA with the (shorter) $\beta 2/\beta 3$ loop in Hera. The conformations of the $\beta 2/\beta 3$ loops differ by up to 8 Å, rendering such an RNA binding mode unlikely for Hera. In contrast, it appears that

the RNA bound across the β -sheet of U1A can be positioned onto Hera_RBD with only minor clashes that may be rectified by slight conformational adjustments (Fig. 2E,F). The U1A-like RNA binding mode would place nucleobases in close proximity to Lys439 in $\beta 1$ and Tyr472 in $\beta 3$ in Hera_RBD. These residues are analogous to the two aromatic residues on the β -sheet surface of canonical RRM. Interactions between the aliphatic side-chain of Lys439 and the aromatic systems of nucleobases are favorable, and in principle these residues could allow for a classical RNA interaction mode similar to U1A.

The Hera_RBD side-chains Lys439 and Tyr472 have their equivalents Tyr407 and Tyr447 in YxiN. Mutagenesis of these tyrosines to alanine did not alter affinity for a 32mer RNA, apparently excluding their involvement in RNA binding (Wang et al. 2006). Together with the differences in the $\beta 1/\alpha 1$ and $\alpha 2/\beta 4$ loop regions noted above, different RNA binding modes of Hera and YxiN may be assumed. This brings up the question as to why YxiN does not use the canonical β -sheet residues for RNA binding, whereas Hera may bind RNA similar to U1A. A close inspection of the RBD structures shows that Tyr447 in YxiN adopts a *gauche plus* rotamer, which is a non-binding competent side-chain conformation that prevents stacking onto the nucleobases (Fig. 2B). The *gauche plus* rotamer of Tyr447 is dictated by the nearby Met442 in the $\beta 2/\beta 3$ loop and is stabilized by Thr440. In contrast, Tyr472 in Hera adopts the *gauche minus* conformation (Fig. 2A), which is the RNA binding competent conformation in all RNA complexes. Two aspects force Tyr472 into the *gauche minus* conformation: First, a Tyr472 *gauche plus* rotamer is impossible in Hera due to sterical hindrance by Leu429 in the small N-terminal domain in Hera_RBD. Second, the small side-chain of Ala467 in Hera does not produce a stereochemical clash with Tyr472, as the equivalent Met442 does for Tyr447 in YxiN. Consequently, Tyr472 in Hera is stabilized in the conformation compatible with a canonical U1A-like RNA binding mode, whereas this binding mode is not possible for YxiN. In summary, a binding mode similar to U1A can be postulated for Hera_RBD, and different RNA binding modes for Hera and YxiN can be rationalized from the structures.

Placement of the RBD in the context of Hera

The RBDs provide DEAD box proteins with high affinity RNA binding of either high specificity for dedicated helicases (Kossen et al. 2002; Linden et al. 2008), or low specificity for helicases that are considered to function as general RNA chaperones (Mohr et al. 2008; Russell 2008; Del Campo et al. 2009). A role of RBDs in positioning neighboring RNA regions for unwinding by the helicase core has been suggested (Diges and Uhlenbeck 2005; Tijerina et al. 2006). While we have previously reported the global localization of the Hera_RBD with respect to the

C-terminal RecA-like domain (Klostermeier and Rudolph 2009), the tracing of the RBD sequence was impossible. We therefore determined the structure of Hera_208-510 that comprises the C-terminal RecA-like domain, the dimerization motif, and the RBD (Fig. 3A). Molecular replacement of Hera_208-510 with Hera_208-419 and Hera_RBD resulted in a model where a monomer is present in the asymmetric unit, and a dimer is produced by crystal symmetry. The dimerization domain in Hera_208-510 is followed by a low complexity linker sequence (GGAPA) that connects the dimerization motif to the RBD. In the structure, this linker sequence traverses the upper part of the RecA_C domain and, as a result, loosely connects the RBD to RecA_C (Fig. 3A, cyan). Only a few polar and hydrophobic, but no hydrogen bond interactions attach the RBD to RecA_C: An electrostatic interaction between Glu227 in RecA_C and Arg426 in the RBD is present, and a total of only 17 van der Waals contacts connect the RBD to RecA_C. Residues participating in hydrophobic interactions are Pro221, Arg223, Gly224, and Arg345 of RecA_C, and residues Arg426, Ser427, Leu428, Thr430, Gly431, Lys463, and Ala465 of Hera_RBD. The buried surface area between the domains covers a mere 435 Å². Notably, most hydrophobic contacts are mediated by residues forming the small globular motif at the N terminus of Hera_RBD (Fig. 3A,B, yellow). The overall positioning of the RBD is with the central β -sheet facing away from RecA_C, leaving a small cleft between the domains. Due to

the likely flexibility of the linker region between the dimerization domain and the RBD, the RBD may adopt several conformations in solution that may be linked to RNA unwinding by the helicase core.

DISCUSSION

In the present work, we have determined the crystal structure of the isolated Hera RNA binding domain. In a second structure, the orientation of Hera_RBD within the framework of the entire helicase was defined. Hera_RBD forms a strongly altered RRM where an entire α -helix is replaced by a loop region. Sequence comparison reveals that this structural motif should be conserved in the D/T group of bacteria. This region is located on the “backside” of the β -sheet and therefore probably not involved in RNA binding, but it may have a structural role.

The N-terminal sequence of Hera_RBD that precedes the RRM is strongly conserved among the D/T DEAD box helicases and will form the same double- β -hairpin structure in all cases, possibly contributing to stabilization of the RRM. The negatively charged motif packs against one end of the β -sheet and could block RNA binding in this region, limiting the conformational space accessible to large RNA substrates. Residues from this region interact with residues of the RRM that are conserved in the D/T group but not in DEAD-box helicases from mesophilic bacteria. N-terminal extensions are also present in YxiN and DbpA, but their structures are unknown. However, these extensions have negatively charged residues in common, which may similarly restrict RNA access to this side of the RRM. An N-terminal extension is also present in the RRM of the U11/U12-65K spliceosomal protein, which binds to a hairpin of the U12 snRNA (Netter et al. 2009). However, in contrast to Hera, this motif of ca. 30 residues folds into a α -helical structure. Deletion of the motif destabilizes the interaction of the U11/U12-65K RRM with RNA, and a structure-stabilizing effect was attributed to this motif (Netter et al. 2009).

Despite the lack of structural data on RNA binding to Hera, a canonical RNA binding mode resembling that of U1A is likely. Sequence comparisons of RRM domains show that deviations from the canonical RNA binding motifs RNP-1 and RNP-2 are common. Hera underscores this observation and adds the possibility that the aromatic side-chain in RNP-2 used for base-stacking can be replaced by aliphatic residues, namely lysine in the *Thermus* and isoleucine in the *Deinococcus* species. Even the aromatic side-chain in RNP-1 is not conserved but can be replaced by valine in *Deinococci* (Fig. 1B). In conclusion, the use of noncanonical RNA binding motifs emphasizes the plasticity of the RRMs not only in eukaryotes but also in prokaryotes.

Comparison of the different poses of RNA in RRM/RNA complexes with Hera_RBD has shown that Hera_RBD could bind RNA in a manner similar to U1A. U1A binds

FIGURE 3. Location of the C-terminal domain in Hera. (A) Ribbon diagram of the refined Hera_208-510 structure. The second protomer of the dimer (gray) is generated by crystal symmetry, placing the Hera_RBDs at a distance of 75 Å. The linker connecting the dimerization domain (DD) to the RBD is shown in cyan and the N-terminal motif of the RBD mediating contact with RecA_C is shown as a yellow tube. (B) Magnification of the contact area between the RBD and RecA_C. Residues of RecA_C contributing to van der Waals interaction with the RBD are shown in pink with their transparent surface. Residues of the RBD contacting RecA_C are shown as yellow or cyan stick models. The polar interaction between Arg426 of the RBD and E227 of RecA_C is indicated by a dashed line.

to a hairpin structure in U1 snRNA and specifically recognizes bases in the loop region. A similar binding mode of Hera to the loop region of hairpin 92 of the 23S rRNA can be envisaged, which would place the sequence 5'-UGUUC-3' across the β -sheet. In this configuration, the stem of the hairpin would project away from the RBD, leaving room for the remainder of the RNA substrate to contact the helicase core. Hera then unwinds a double-stranded region 5' to the hairpin structure, and the RBD could help to align the RNA substrate for unwinding by the helicase core. In contrast to YxiN which displays a high specificity for hairpin 92 in 23S rRNA, Hera is promiscuous and also binds to RNase P RNA (Linden et al. 2008). The possible positioning function combined with substrate promiscuity would thus be analogous to the role of the basic CTDs preferentially interact with structured RNAs, and the helicase unwinds adjacent duplexes that are not tightly interacting with the structured part of the RNA (Tijerina et al. 2006).

While the dynamic interplay between the helicase core and the RBD is currently unknown, the crystal structures described here shed light on the juxtaposition of the Hera domains in the complete structure. The structure of the Vasa helicase core in the closed conformation (Sengoku et al. 2006) was used as a template to assemble a model for the dimeric Hera helicase from the individual domains (Fig. 4A; Rudolph et al. 2006; this work). The RNA co-crystallized with Vasa is short and only contacts the helicase core. The overall RNA binding mode of the helicase core is conserved and, if Hera_RBD binds RNA similarly to U1A, two pieces of RNA can be placed on the dimer: one bound across the core domain interface, and the second bound to the RBD (Fig. 4A). The directionality of these RNA molecules is the same, and the 5'-end of the core-bound RNA points toward the RBD. Electrostatic surface potential calculations of the Hera model identified an elongated, positively polarized region generated by arginine residues 225, 246, and 314 that starts 5' of the poly-U binding site of Vasa and stretches via RecA_C toward the RBD. A positively charged ridge is present close to the RBD, formed by arginines 223, 345, 347, and 348 (Fig. 4B). These residues were suggested as possible RNA interacting side-chains (Klostermeier and Rudolph 2009), and passing of the RNA over this ridge on RecA_C to the RNA interacting with the RBD could be envisaged. There is also positive potential at the 3' end of the bound RNA, possibly extending the RNA binding surface on RecA_N. The entire path could mark an elongated, unspecific ssRNA binding site. An extended binding site involving the helicase core and a C-terminal domain is observed in a recent crystal structure of Mss116 in complex with U10 ssRNA (Del Campo and Lambowitz 2009). The RNA bound to

FIGURE 4. Possible binding site on Hera for large RNA molecules. (A) Construction of a model for the Hera dimer by superposition of the Hera_N (PDB-ID 1gxs) and Hera_208-510 (this work) structures with the Vasa-RNA complex. The domains are individually colored and labeled. The view is rotated by 45° about the y -axis compared to Figure 3A and the RBD faces the viewer. (B) The surface electrostatic potential of the Hera dimer shows a positively polarized trace between the known RNA binding site on the helicase core and the RBD that could mark the path of a large RNA substrate (marked in orange). The RNAs were taken from the superposition with Vasa and U1A and are shown as stick models.

Mss116 has the same orientation as proposed for Hera and the 5'-end contacts a four-helix bundle C-terminal to the helicase core. In Hera, at least 25 nucleotides (nt) of ssRNA could be spanned across a monomer if the RNA followed the positive path. The dimer may therefore interact with 50 nt or more, possibly in the context of larger RNA substrates.

MATERIALS AND METHODS

Protein production, purification, and crystallization

Hera_208-510 was cloned, produced, purified, crystallized, and MAD-phased as described (Rudolph et al. 2009). As the C-terminal 90 residues could not be traced, the region coding for Hera_RBD was cloned separately into pETM30. The protein was overproduced in *E. coli* Rosetta(DE3) in autoinducing medium (Studier 2005), and cells were disrupted in 50 mM Tris/HCl at pH 7.5 and 500 mM NaCl using a microfluidizer. The supernatant was purified via affinity chromatography on GSH-sepharose in the same buffer, and eluted with buffer containing 20 mM glutathione. After hydrolysis with TEV protease (4°C, overnight), the remaining fusion protein and the His₆-GST tag were removed using Ni²⁺-NTA agarose (50 mM Tris/HCl at pH 7.5, 500 mM NaCl, 20 mM imidazole), followed by S75 size-exclusion chromatography (50 mM Tris/HCl at pH 7.5, 500 mM NaCl). Hera_RBD was concentrated to 1.4 mM in 50 mM Tris/HCl at pH

7.5, 500 mM NaCl, and diluted threefold with water prior to crystallization. Crystals were obtained after two weeks at 22°C via vapor diffusion in sitting drops by mixing equal volumes (0.3 μ L) of protein with reservoir solution consisting of 0.1 M Na-cacodylate at pH 6.5, 16%–18% PEG8000, 0.3 M–0.4 M Zn(OAc)₂. A crystal was derivatized using a 1-min soak with 0.25 M KI in reservoir solution. Prior to data collection, crystals were vitrified in paraffin oil using the hyper-quenching technique (Warkentin and Thorne 2007).

Data collection, structure determination, and refinement

Hera_RBD crystals are of space group P4₁2₁2 and contain one molecule per asymmetric unit. Data were collected at 100 K and reduced with XDS (Kabsch 1993) and SADABS (Bruker). Native data were collected on a Pilatus detector on SLS beamline X06SA to a resolution of 1.8 Å. KI derivative data were collected to a resolution of 2.14 Å on a MAR-CCD225 detector on beamline X06DA using a wavelength of 1.8 Å, where the calculated $f' = -1.1e^-$ and $f'' = 8.8e^-$. For SIRAS phasing, the anomalous and SIR differences were extracted by XPREP (Bruker) using data to a resolution of 2.5 Å. SHELXD was used to determine a three-atom substructure. Enantiomorph selection and density modification including phase extension to 1.8 Å resolution were performed with SHELXE, which also provided a first auto-traced polyaniline model of 73 out of 87 residues. The three iodides bind to the side-chain of R476 (8.7 σ), between the side-chains of R449 and P443 (8.1 σ), and to the main-chain amide groups of L497 and L498 (5.5 σ). It should be noted that exhaustive molecular replacement searches using the MAD electron density (Klostermeier and Rudolph 2009), all available RRM-like models (including the YxiN homolog), or models calculated de novo from the Hera_RBD sequence using ROSETTA (Das and Baker 2008) were unsuccessful. Also unsuccessful were attempts to MAD-phase the crystals using data from a KBr soak or chemically modifying the two tyrosine side-chains in Hera_RBD using elementary iodine. The Hera_208–510 structure was determined via molecular replacement using the Hera_208–419 (Klostermeier and Rudolph 2009) and Hera_RBD structures as starting models with PHASER (McCoy et al. 2007). Models were built in COOT (Emsley and Cowtan 2004) and refined with PHENIX (Zwart et al. 2008). Five percent of reflections were reserved for R_{free} cross-validation in all structures (Brünger 1992). The C-terminal 12 residues (sequence EIPSRTRRPARA) were omitted from the final models due to lack of electron density. Statistics are summarized in Table 1. Figures were created with Bobscript (Esnouf 1997), Raster3D (Merritt and Murphy 1994), PyMol (<http://www.pymol.org>), or ESPRIPT (Gouet et al. 1999).

DATA DEPOSITION

The coordinates and structure factors have been deposited in the Protein Data Bank (accession codes 3I31 and 3I32).

SUPPLEMENTAL MATERIAL

Supplemental material can be found at <http://www.rnajournal.org>.

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