***Escherichia coli* DbpA is an RNA helicase that requires hairpin 92 of 23S rRNA**

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***Escherichia coli* DbpA is a member of the DEAD/H family of proteins which has been shown to have robust ATPase activity only in the presence of a specific region of 23S rRNA. A series of bimolecular RNA substrates were designed based on this activating region of rRNA and used to demonstrate that DbpA is also a non-processive, sequence-specific RNA helicase. The high affinity of DbpA for the RNA substrates allowed both single and multiple turnover helicase assays to be performed. Helicase activity of DbpA is dependent on the presence of ATP or dATP, the sequence of the loop of hairpin 92 of 23S rRNA and the position of the substrate helix with respect to hairpin 92. This work indicates that certain RNA helicases require particular RNA structures in order for optimal unwinding activity to be observed.**

Keywords: *E.coli* 23S rRNA/processivity/RNA-binding protein

Introduction

The rearrangement of RNA secondary structures is a central requirement for many aspects of RNA metabolism. For instance, the spliceosome creates and disrupts interactions between snRNAs and the pre-mRNA in order to target the appropriate splice sites (Will and Luhrmann, 1997). However, RNA secondary structures can be extremely stable, with helices >9 bp having half-lives of the order of hours (Xia *et al.*, 1998), and thus cannot be left to unwind on their own if cellular processes are to proceed at a reasonable rate. This problem is circumvented by a family of proteins known as DEAD/H proteins that use the energy of ATP hydrolysis to disrupt stable RNA secondary structures (Schmid and Linder, 1992). DEAD/H proteins participate in mRNA and rRNA processing (Lagerbauer *et al.*, 1998), mRNA transport and RNA degradation (Daugeron and Linder, 1998; Luking *et al.*, 1998) and translation initiation (Blum *et al.*, 1992; Iost *et al.*, 1999; Svitkin *et al.*, 2001), and several of them have been shown to be essential for cell survival (Chuang *et al.*, 1997; Kressler *et al.*, 1997, 1998; de la Cruz *et al.*, 1998; Colley *et al.*, 2000). DEAD/H proteins are defined by seven conserved motifs that are required for ATP binding and hydrolysis and RNA unwinding (Gorbalenya and Koonin, 1993; Fuller-Pace, 1994). Most DEAD/H proteins also contain unique N- and/or C-terminal extensions that may mediate their interactions with other proteins or specific

RNA sequences (Fuller-Pace and Lane, 1992; Wang and Guthrie, 1998). *In vivo*, DEAD/H proteins are found generally as members of large multiprotein complexes and often act to rearrange a single RNA helix in a complex pathway. The other members of these multiprotein complexes are believed to be responsible for directing DEAD/H proteins to their appropriate substrates (Luking *et al.*, 1998).

Purified DEAD/H proteins are difficult to study biochemically since many lose much of their substrate specificity and activity in the absence of their accessory proteins (Abramson *et al.*, 1988; Wang *et al.*, 1998). Although most DEAD/H proteins tested demonstrate an RNA-activated ATPase activity, these RNA activators need not bear any relationship to the *in vivo* RNA target (Pause and Sonenberg, 1992; Shuman, 1992; Morgenstern *et al.*, 1997). A few DEAD/H proteins display an ATP-dependent RNA helicase activity, but again non-biologically relevant bimolecular RNA substrates were used (Shuman, 1993; Wang *et al.*, 1998; Yu and Owtrim, 2000). For example, the DEAD/H protein PRP16 associates with the spliceosome and is required for the second chemical step of splicing (Staley and Guthrie, 1998), probably in a proofreading pathway that ensures branch point fidelity (Burgess *et al.*, 1990; Burgess and Guthrie, 1993). *In vitro*, PRP16 hydrolyzes ATP at a rate of 6.9×10^{-4} /min in the presence of poly(A) RNA, a rate that is clearly too slow to be biologically relevant (Wang *et al.*, 1998). PRP16 has been shown to disrupt the U4–U6 duplex in the presence of ATP (Wang *et al.*, 1998); however, this activity has no bearing on the biological role of PRP16 (Umen and Guthrie, 1995) since the U4–U6 interaction is disrupted prior to the second step of splicing at which the protein acts. The difficulty in identifying native substrates hinders the study of purified DEAD/H proteins, yet their ubiquity and necessity for survival makes them an obvious target of biochemical analysis.

The *Escherichia coli* protein DbpA is the first example of a DEAD/H protein that has its ATPase activity stimulated by a specific RNA *in vitro* (Fuller-Pace *et al.*, 1993). DbpA is activated by a region of 23S rRNA that contains part of the peptidyltransferase center of the ribosome (Nicol and Fuller-Pace, 1995). Virtually no ATPase activity is observed in the presence of other RNAs. Investigation of the RNA sequence and structural requirements for RNA-activated ATPase of DbpA has revealed that this activity is dependent on a sequence-specific interaction with hairpin 92 in 23S rRNA and a non-sequence-specific interaction with a single-stranded region either 3' or 5' of the hairpin (Tsu *et al.*, 2001). Even though the function of DbpA is not yet known, its lack of activation by intact ribosomes indicates that it is likely to be involved in an aspect of ribosome maturation (Tsu and

Uhlenbeck, 1998). DbpA and its bacterial homologs contain the seven conserved motifs common to all DEAD/H proteins plus a unique 75 amino acid C-terminal extension (Kossen and Uhlenbeck, 1999). It is believed that this extension is responsible for conferring RNA specificity on DbpA by binding to hairpin 92 (Tsu *et al.*, 2001). In order for DbpA to be an appropriate model system for studying the mechanism of DEAD/H proteins, it remained to be established that it indeed has helicase activity. Previous reports have suggested that DbpA either has no helicase activity (Pugh *et al.*, 1999), is an ATP-independent unwinding protein (Boddeker *et al.*, 1997) or is a weak, non-sequence-specific RNA helicase (Henn *et al.*, 2001). However, here we show that DbpA is a robust sequence-specific ATP-dependent RNA helicase whose activity depends upon the presence of hairpin 92 and precisely positioned substrate helices.

Results

Helicase substrate design and initial characterization of helicase activity

Several factors dictated the design of RNA helicase substrates for DbpA. The first was the inherent RNA specificity exhibited by DbpA. Since the ATPase activity of DbpA requires hairpin 92 of 23S rRNA (Tsu *et al.*, 2001), all potential helicase substrates must incorporate this hairpin. Most helicases require a region of single-stranded nucleic acid prior to the start of the helix in order to disrupt a duplex efficiently (Shuman, 1993; Ali *et al.*, 1999), therefore this feature had to be incorporated into the RNA substrates as well. Helicases can unwind nucleic acids in either a 3' to 5' or 5' to 3' direction with respect to the translocating strand (Rozen *et al.*, 1990; Ali *et al.*, 1999; Paolini *et al.*, 2000). Two families of helicase substrates were designed to test both possibilities. Finally, the degree of processivity of DbpA had to be considered. A low degree of processivity is exhibited by eIF4A, which is only capable of unwinding helices with a free energy of less than -18 kcal/mol (~ 12 bp) when acting on its own (Rogers *et al.*, 1999). Therefore, substrates for DbpA were designed to have short RNA helices to ensure detection of even non-processive helicase activity.

Consideration of all of these factors led to the creation of the initial helicase substrate shown in Figure 1A. This substrate is based on an RNA cofactor that maximally activates the ATPase of DbpA (Tsu and Uhlenbeck, 1998), and is a bimolecular complex consisting of a 75 nucleotide RNA containing hairpin 92 and a ^{32}P -labeled nine base oligonucleotide annealed to the 75mer four bases 5' of the hairpin. The single-stranded region between hairpin 92 and the RNA helix was included because the co-crystal structures of the helicases PcrA and hepatitis C virus (HCV) NS3 complexed with DNA (Kim *et al.*, 1998; Velankar *et al.*, 1999) revealed that both proteins bound to a short region of single-stranded nucleic acid. A bimolecular helicase substrate was designed containing a 9 bp duplex that has a calculated ΔG of -16.8 kcal/mol at 37°C based on nearest neighbor contributions to helix stability (Xia *et al.*, 1998). The complex has a calculated dissociation rate, k_{off} , of $4.55 \times 10^{-6}/\text{min}$ assuming the well-established rate of diffusion limited strand

association of $10^8/\text{M}/\text{min}$ (Xia *et al.*, 1998). Experiments confirmed that under the conditions of the assay, the substrate helix remains fully associated for at least 90 min (data not shown). However, if only 3 bp of this helix were disrupted by DbpA, the stability of the remaining 6 bp is expected to be reduced sufficiently to cause rapid helix dissociation (calculated $k_{\text{off}} = 740/\text{min}$). Thus, little or no processivity would be needed for DbpA to disrupt the 9 bp substrate duplex. This substrate therefore had all the features to provide a sensitive test for helicase activity of DbpA.

Helicase assays are generally performed under conditions where a displaced oligonucleotide cannot reanneal to its complement during the time course of the reaction. This is done most commonly by using very low nucleic acid substrate concentrations in conjunction with enzyme concentrations that are high enough to drive binding. As a result, helicase assays generally are performed under conditions of enzyme excess over substrate, allowing only single turnover reactions to be observed. Although the high affinity of DbpA for its RNA substrate potentially could permit substrate excess experiments, initial helicase reactions were performed in enzyme excess following convention (Hirling *et al.*, 1989; Levin and Patel, 1999; Rogers *et al.*, 1999). Preliminary experiments revealed that in order to prevent spontaneous helix formation in a 90 min assay at 25°C , the concentration of each component of the bimolecular helicase substrate must be below 5 nM (data not shown). Therefore, initial helicase assays were performed using 1 nM substrate. Under these conditions, the k_{on} was determined to be $0.006/\text{min}$ so that as the oligonucleotide is displaced by DbpA over the course of a 45 min assay, little reannealing will occur.

Helicase assays were performed by mixing 1 nM bimolecular RNA substrate with a series of increasing DbpA concentrations in the presence and absence of different nucleoside triphosphates and analyzing the products on a non-denaturing gel (Figure 1B). In the absence of ATP, the mobility of the substrate is retarded in the gel due to the formation of a complex with DbpA. These data can be fit to a simple binding curve with a $K_D = 22$ nM. This value is similar to the apparent binding affinities for the 153mer RNA defined by both the ATPase and gel shift assays (Tsu and Uhlenbeck, 1998; K.J.Polach, unpublished observations). In the presence of saturating concentrations of ATP-Mg $^{2+}$, increasing concentrations of DbpA resulted in displacement of the ^{32}P -labeled nine base oligonucleotide from the RNA substrate (Figure 1B and C, left panel). At the highest concentration, DbpA disrupted $>95\%$ of the RNA duplex. While similar results were obtained in the presence of dATP, no activity was observed when AMPPNP, ADP or other NTPs were included in the reaction, despite the fact that RNA binding can occur in the presence of these other nucleotides (Figure 1B).

The helicase assay was then performed at saturating concentrations of DbpA (300 nM) and monitored as a function of time. The data in Figure 1C, right panel, show that the helicase activity generally follows first-order kinetics and that strand displacement proceeds to $\sim 85\%$. This demonstrates that the observed helicase activity is a time-dependent process and not a strand displacement reaction resulting from increasing protein concentrations.

Fig. 1. Helicase activity of DbpA. (A) The 153 nucleotide fragment of 23S rRNA which activates DbpA (left) and the bimolecular helicase substrate F1 between bases 2532 and 2606 of 23S rRNA and a 5'-³²P-labeled 9mer. (B) Nucleotide dependence of helicase activity. Lane 1, 75mer marker (rRNA bases 2532–2606); lane 2, 5'-³²P-labeled 9mer; lane 3, F1; lanes 4–8, 9–13 and 14–18, DbpA titrations of 15, 50, 150 and 600 nM DbpA, incubated for 30 min; lanes 19–23, 600 nM DbpA with 1 mM of the indicated NTP in each reaction. (C) Helicase activity as a function of DbpA concentration with 1 nM RNA and 2 mM NTP-Mg²⁺ after 30 min incubation (left) or as a function of time at 300 nM DbpA (right). No NTP (circles), 2 mM ATP (upright triangles), 2 mM dATP (squares), 2 mM AMPNP (diamonds) or 2 mM ADP (inverted triangles).

Order of addition experiments showed no dependence on the rate of unwinding as a function of pre-formation of either a DbpA:ATP or DbpA:RNA complex.

Processivity and kinetics

Having established that DbpA can support helicase activity by disrupting short RNA helices 5' of hairpin 92,

Fig. 2. DbpA is a non-processive helicase. (A) RNA oligonucleotides of varying stabilities annealed to a 75 nucleotide fragment of rRNA to create 3' helicase substrates T1–T3. Helicase activity of T-1 (circles), T-2 (squares) and T-3 (diamonds) as a function of increasing concentrations of DbpA, with 2 mM ATP-Mg²⁺ and 1 nM RNA. (B) RNA oligonucleotides of varying stabilities that were complementary to the region 5' of hairpin 92 were annealed to a 51 nucleotide RNA. Helicase activity of F-2 (circles), F-3 (triangles) and F-4 (squares) as a function of DbpA concentration with 2 mM ATP-Mg²⁺ and 1 nM RNA.

the next step was to determine whether DbpA could disrupt helices 3' of the hairpin. New helicase substrates were created based on the previous RNA substrate that contained RNA duplexes 3' of hairpin 92 (Figure 2A, left panel). In the presence of ATP, DbpA efficiently disrupted these substrate duplexes (Figure 2A, right panel), suggesting that DbpA is a bidirectional helicase. To test whether DbpA was a processive helicase as well, substrates were constructed that contained a more stable 15 bp helix ($\Delta G = -29.8$ kcal/mol) either 3' or 5' of hairpin 92 (Figure 2A and B). In order for such a stable helix to be disrupted, DbpA would have to unwind at least 9 bp in order for fast, spontaneous helix dissociation to occur. The results show that DbpA is unable to disrupt a long helix either 5' or 3' of hairpin 92. However, DbpA can efficiently disrupt shorter RNA helices located in the same region, demonstrating that the lack of unwinding activity is indeed due to the stability of the long substrate helix and not the position of the helix. This result indicates

that despite its high affinity for its RNA substrate, DbpA is not a processive helicase.

In order to perform a more extensive analysis of the helicase activity of DbpA, a smaller substrate was developed (Figure 3A). This bimolecular RNA was based on the 32mer RNA that previously has been demonstrated to bind DbpA with high affinity and stimulate its ATPase activity to maximal levels (Tsu *et al.*, 2001). When annealed to the same 9mer that was used in the previous experiments, the 32mer can act as a bimolecular helicase substrate for DbpA. In the absence of ATP, this minimal RNA helicase substrate is bound by DbpA with a $K_D = 13$ nM as measured by the gel-shift assay. In the presence of saturating levels of ATP, the substrate helix is ~80% disrupted in a time course assay using 300 nM DbpA (Figure 3B). Initial rates for helicase activity were measured at several concentrations of DbpA. Analysis of four separate data sets in an Eadie–Hofstee plot (Figure 3C) gave a $V_{max} = 0.16 \pm 0.05/\text{min}$ and a

Fig. 3. Kinetics of helicase activity. (A) A minimal RNA helicase substrate consisting of the 32mer minimal RNA cofactor and an annealed complementary oligonucleotide. (B) Time course of helicase assay with 1 nM RNA and without (circles) or with (squares) 2 mM ATP. (C) Initial rates of helicase activity at eight concentrations of DbpA (3, 10, 15, 20, 30, 75, 100 and 300 nM) were used to construct an Eadie-Hofstee plot fit to $K_M(\text{DbpA})$ of 19.8 ± 4.5 nM and $V_{\max}$ of $0.16 \pm 0.05/\text{min}$.

$K_M = 19.8 \pm 4.5$ nM. This K_M of protein for RNA agrees well with the K_D for RNA and DbpA derived from the gel-shift binding assays for this substrate.

The high affinity of DbpA for the bimolecular RNA substrate makes it possible to determine the k_{cat} for helicase activity under multiple turnover conditions. Under the high substrate concentrations required in order to observe multiple turnovers, reannealing of the labeled nine nucleotide oligomer to the 32mer will occur, reducing the apparent unwinding rate. To prevent this, a 25-fold excess of non-radioactive 9mer was included in the reactions as a trap to prevent the 32mer from reannealing to the displaced radiolabeled 9mer. In addition, the coupled pyruvate kinase-lactate dehydrogenase system

(Bessman, 1963) was added initially to the multiple turnover reactions to prevent the accumulation of inhibitory concentrations of ADP during the reaction. However, rates of helicase activity in the presence and absence of the regeneration system showed no significant difference and the system was omitted from further reactions. The average $V_{\max}$ for helicase activity determined from several multiple turnover assays is $0.14 \pm 0.02/\text{min}$, with each molecule of DbpA turning over an average of 5.3 times during an assay.

Structural requirements for helicase activity

DbpA is unique among DEAD/H proteins in that only certain RNAs are capable of stimulating its ATPase activity (Nicol and Fuller-Pace, 1995; Tsu and Uhlenbeck, 1998). In order to investigate whether similar RNA sequence requirements are observed for the helicase activity of DbpA, a double mutation (G2553U, U2555A) known to abolish ATPase activity of DbpA (Tsu *et al.*, 2001) was introduced into hairpin 92 of the minimal bimolecular helicase substrate (Figure 4A). This substrate mutation dramatically reduced the ability of DbpA to bind the substrate, with only a small fraction of the substrate forming a high molecular weight aggregate at concentrations >250 nM protein (data not shown). As shown in Figure 4B, this substrate showed no detectable helicase activity at all concentrations of DbpA, suggesting that the high molecular weight aggregate is not catalytically active. In a second experiment, the effect of the sequence of the substrate helix on the helicase activity of DbpA was investigated by introducing a triple mutation (C2538U, C2540U, A2542U) into the single-stranded region of the 32mer RNA and annealing a complementary oligonucleotide to produce the bimolecular helicase substrate (Figure 4A). DbpA bound this bimolecular RNA and disrupted the RNA helix as well as it did the original RNA substrate (Figure 4B). These results support the hypothesis that DbpA binds to the RNA substrate by recognizing specific elements within hairpin 92, and also makes non-sequence-specific contacts with the RNA 5' of the hairpin.

Most known DNA and RNA helicases prefer to unwind nucleic acid duplexes which are preceded by a region of single-stranded nucleic acid (Shuman, 1993; Ali *et al.*, 1999; Rogers *et al.*, 2001). The RNA substrate included a region of four single-stranded bases between the substrate helix and hairpin 92. In order to investigate whether this region of single-stranded RNA was necessary for activating helicase activity, a series of helicase substrates were designed having helices of similar thermodynamic stabilities placed at varying distances 5' of hairpin 92 (Figure 5A, left panel). Recent work by Rogers *et al.* (2001) demonstrates that the rate and amplitude of helix unwinding by an RNA helicase are determined by the overall stability of an RNA duplex rather than its length or sequence. Therefore, all duplexes had thermodynamic stabilities less than that of duplex F-1 in order to bias them towards disruption by DbpA. Even though DbpA bound each of the new helicase substrates with affinities similar to the original bimolecular RNA (K_D s ranged from 7 to 21 nM, data not shown), helices were only fully disrupted when at least four bases intervened between hairpin 92 and the first base pair of the substrate helix (Figure 5A, right panel). Shifting the substrate helix to within three bases of

Fig. 4. (A) Two mutant helicase substrates. (B) Helicase activity of unmodified substrate (circles), hairpin mutant (diamonds) and helix mutant (squares) assayed at increasing concentrations of DbpA, 2 mM ATP-Mg²⁺ and 1 nM RNA.

the hairpin reduced helicase activity by 2-fold, and helices beginning within two bases of hairpin 92 were not disrupted.

To test whether a similar spacing requirement existed in order for DbpA to unwind helices 3' of hairpin 92, a set of substrates containing helices of similar thermodynamic stabilities was created (ΔG between -16.5 and -17.5 kcal/mol) (Figure 5B, left panel). Although DbpA can disrupt helices 3' of hairpin 92, this activity is dependent upon the presence an 11 nucleotide region of single-stranded RNA between the duplex and the hairpin (Figure 5B, right panel). Reduction of the single-stranded region to 10 bases results in a 3-fold drop in the level of helix disruption. When the helix is moved to within five bases of hairpin 92, no unwinding is observed. These results demonstrate that optimal activation of the 3' helicase activity of DbpA requires a longer region of single-stranded RNA than was observed for the 5' helicase activity.

Discussion

DbpA is unique among DEAD/H proteins in that a region of 23S rRNA specifically activates the ATPase activity of the purified protein (Fuller-Pace *et al.*, 1993; Tsu and Uhlenbeck, 1998). This specificity indicates that DbpA is likely to interact with this region of rRNA *in vivo*, and thus is a component of the biologically relevant RNA target of the protein. It was possible that DbpA required other RNA elements apart from the previously identified hairpin 92

(Tsu *et al.*, 2001) in order for helicase activity to be observed. To that end, we performed helicase assays using rationally constructed RNA helicase substrates that incorporated elements needed for DbpA binding and ATPase stimulation in particular, as well as elements needed for the unwinding activity of other RNA and DNA helicases. In the presence of ATP, DbpA completely and efficiently disrupted the bimolecular RNA substrates created from this design strategy in both protein-dependent and time-dependent assays. The high affinity of DbpA for these RNA substrates (in the nM range) allowed for the performance of kinetic studies. These studies give insight into the nature of the interaction of DbpA with an RNA substrate having biological relevance, and also allow for comparisons to be made between DbpA and other biochemically characterized DEAD/H proteins.

Both single turnover (protein excess) and multiple turnover (substrate excess) helicase assays were performed using the bimolecular RNA helicase substrates. These experiments show that DbpA is indeed capable of unwinding a short RNA helix without being modified or losing its activity in the process. Several crystal structures of DEAD/H proteins show that this family of proteins all have similar structures containing the seven conserved domains and so are likely to act via similar mechanisms (Korolev *et al.*, 1997; Kim *et al.*, 1998; Velankar *et al.*, 1999; Story *et al.*, 2001). It is possible, based on these results, that all DEAD/H proteins are catalytic helicases, but that the poor RNA affinities of these purified proteins *in vitro* have made this fact difficult to demonstrate biochemically.

The comparison of the rates of multiple and single turnover helicase activity of DbpA allowed for some preliminary characterization of the mechanism of helicase activity. The rates of unwinding, 0.16/min in protein excess and 0.14/min in RNA excess, are similar, indicating that release of the unwound RNA is not a rate-limiting step for helicase activity. This rate of unwinding compares favorably with the rates measured for other DEAD/H family helicases. The DEAD/H protein PRP16 unwinds the U4-U6 duplex at an initial rate of 0.11/min (Wang *et al.*, 1998) and the prototypical DEAD/H protein eIF4A unwinds RNA helices with a maximal initial rate of 13 fmol/min (Rogers *et al.*, 1999). The processive RNA helicases unwind RNA with much higher rates: vaccinia virus helicase NPH-II unwinds RNA at a rate of 3.3 ± 0.5 /min (Jankowsky *et al.*, 2000) under conditions of protein excess, and excess HCV NS3 unwinds DNA at a maximal initial rate of 21/min (Levin and Patel, 1999), indicating that there may be significant differences in the way these two types of proteins disrupt nucleic acid structures.

The ability of DbpA to unwind a substrate helix is dependent upon the position of the duplex with respect to hairpin 92. Even though DbpA bound all of the RNA substrates tested with similar affinities and all stimulated the ATPase activity of the protein, only a few of the RNA helices were unwound. At least four bases of single-stranded RNA must intervene between hairpin 92 and a substrate helix 5' of the structure in order for complete unwinding to be observed. This requirement for a region of single-stranded RNA prior to the substrate helix for full activation of helicase activity has been observed for

Fig. 5. Helix position changes the helicase activity of DbpA. **(A)** Helicase substrates with helices at different distances 5' of hairpin 92 were assayed for unwinding activity. Helicase activity of F-1 (circles), F-5 (squares), F-6 (diamonds) and F-7 (triangles) at increasing concentrations of DbpA, 2 mM ATP-Mg²⁺ and 1 nM RNA. **(B)** Helicase substrates with helices at different distances 3' of hairpin 92 were assayed for unwinding activity. Helicase activity of T-2 (triangles), T-3 (circles), T-4 (squares) and T-5 (diamonds) at increasing concentrations of DbpA, 2 mM ATP-Mg²⁺ and 1 nM RNA.

several other helicases, including the prototypical DEAD/H protein eIF4A (Rogers *et al.*, 1999), the vaccinia virus helicase NPH-II (Shuman, 1993) and HCV NS3 helicase (Tai *et al.*, 1996). The co-crystal structures of both PcrA (Velankar *et al.*, 1999) and HCV NS3 (Kim *et al.*, 1998) show these proteins bound to 4–6 bases of single-stranded nucleic acid. This finding is unique to DbpA in that the observed spacing requirement is in relation to a specific RNA structure, hairpin 92, that is necessary for both protein binding and activity.

The data presented here correlate well with the model proposed by Tsu *et al.* (2001) for the interaction of DbpA with its RNA substrate. In this model, the unique C-terminal domain of DbpA binds specifically and tightly to hairpin 92 of 23S rRNA, thereby conferring sequence specificity, but not activating the ATPase. The N-terminal domain, which contains the seven conserved DEAD/H motifs, is believed to interact with a nearby single-stranded region of RNA in a non-specific fashion and activates the ATPase. The helicase assays reported here

demonstrate that mutations in hairpin 92 abolish the high affinity interaction of DbpA with the bimolecular RNA substrates, but mutations in the 5' extension sequence remain active. Thus, the specificity of the helicase activity is the same as the ATPase activity.

There are two possible explanations for the different spacing requirements for optimal displacement of substrate helices 5' and 3' of the hairpin (four and 11 bases, respectively). The first is that DbpA is a bidirectional helicase capable of unwinding helices in either a 5' to 3' or a 3' to 5' direction. The variability in spacing requirement could then be explained by the different orientations DbpA must assume relative to the substrate RNA helix in order to disrupt it. The other possibility is that DbpA only unwinds RNA in the 3' to 5' direction with respect to the translocating strand. In this case, the DEAD/H domains of DbpA would only bind the single strand-double strand RNA junction 3' of the substrate helix in order for strand displacement to occur. The 11 bases of single-stranded RNA required to disrupt helices 3' of hairpin 92 would

then be needed to allow the RNA to wrap around to facilitate binding of the DEAD/H domains to the appropriate site.

A previous attempt to demonstrate that DbpA can act as a helicase by unwinding RNA duplexes near hairpin 92 of rRNA was unsuccessful (Pugh *et al.*, 1999) probably because the helicase substrate used in the assays contained a helix that was too stable and did not include a region of single-stranded RNA between the substrate helix and hairpin 92. A more recent report showing that DbpA is able to unwind long RNA helices lacking hairpin 92 at very high protein concentrations (Henn *et al.*, 2001) appears to contradict our observation that long helices are not unwound. This dramatic difference in specificity and processivity can be understood by the model. The high affinity interaction of the C-terminal domain with hairpin 92 may act to 'anchor' DbpA to a specific region of RNA, preventing the protein from processing along the RNA strand and thereby disrupting long helices. When hairpin 92 is absent, the N-terminal DEAD/H domains of DbpA may still maintain the weak, non-specific affinity for the RNA substrate that is observed with other DEAD/H proteins such as eIF4A (Lorsch and Herschlag, 1998), PRP16 (Wang *et al.*, 1998) and PRP28 (Strauss and Guthrie, 1994). Indeed, gel-shift binding assays performed with DbpA and the 153mer reveal the formation of supershifted protein-RNA complexes at concentrations of DbpA exceeding 220 nM that are the result of additional molecules of DbpA binding non-specifically (K.J.Polach and O.C.Uhlenbeck, in preparation). Since we observed that these non-specific complexes are not catalytically active, the weak processive helicase activity observed by Henn *et al.* (2001) at DbpA concentrations >0.3 μ M can be understood as a combination of a low affinity, non-processive unwinding activity of DbpA in concert with the non-specific single-stranded binding activity of the protein, which prevents the helix from reforming.

The experiments presented here demonstrate that DbpA is an ATP-dependent non-processive RNA helicase that requires hairpin 92 of 23S rRNA for optimal activity. In the context of the RNA substrates investigated here, DbpA resembles the non-processive RNA helicase eIF4A more closely than it does the processive RNA helicases NPH-II and HCV NS3. DbpA is thought to be involved in ribosome biogenesis in *E.coli*, and 23S rRNA does not contain long regions of double-stranded RNA, but rather is made up of short regions of secondary structure and long-range tertiary interactions. It is likely that DbpA disrupts short regions of inappropriately folded rRNA during ribosome biogenesis, and so does not need to be processive in the context of its biologically relevant substrate. However, the actual RNA substrate of DbpA is still unknown; as long as minimal spacing requirements were met, DbpA could possibly disrupt any helix near hairpin 92 in the pre-ribosomal complex.

Materials and methods

Materials

DbpA was expressed and purified by the protocol of Tsu and Uhlenbeck (1998). DbpA was concentrated into storage buffer [20 mM MOPS pH 6.8, 50 mM NaCl, 1 mM dithiothreitol (DTT) and 50% glycerol] and stored at -80 or -20°C . DbpA concentration was determined by the

absorbance at 280 nm using a molar extinction coefficient of 27 680/M/cm calculated from the amino acid sequence in 6.0 M guanidine HCl.

Plasmid PKK 3535 containing the DNA for domain V of *E.coli* 23S rRNA was amplified by PCR using primers 5'-TAATACGACTCATATAGGAGGTCCCAAGGGTATGGCTGTT-3' containing the T7 promoter, and 5'-GAAGTGTCTCAGCAGCTTCTAAACCCAGCTCG-3' to generate a T7 transcription template for R75, the component of the bimolecular helicase substrate consisting of nucleotides 2532–2606 of 23S rRNA. After transcription with T7 RNA polymerase (Sampson and Uhlenbeck, 1988), the 75mer was purified by denaturing 10% acrylamide–7 M urea gel electrophoresis. The 51mer RNA was prepared in a similar fashion. Short RNA oligonucleotides were chemically synthesized by Dharmacon Research, 5'- ^{32}P -labeled with T4 polynucleotide kinase (from New England Biolabs) and [γ - ^{32}P]ATP. Templates for the transcription of the 32mer RNA were made from two DNA oligonucleotides with sequences: 5'-TAATACGACTCACTATAGGAGGTCCCAAGGGTATGGCTGTTTCGCCATTT-3' and 5'-AAATGGCGAACAGCCATACCCTTGGGACCTCTATAGTGAGTCGTATTA-3' (Milligan and Uhlenbeck, 1989). The mutant 32mer RNAs were also chemically synthesized by Dharmacon Research.

Bimolecular RNA substrates were formed by annealing R75, R51 or R32 and its derivatives to the desired short oligonucleotide. Annealing conditions were as follows: 1.5 μ M R75, R51 or R32, 1 μ M short oligonucleotide (final concentration, including the radiolabeled oligonucleotide), 2.7 μ Ci of ^{32}P -labeled oligonucleotide, 50 mM HEPES pH 7.5 and 50 mM KCl in a total volume of 5 or 10 μ l. The reaction was incubated at 95°C for 60 s, followed by 3 min at 65°C . MgCl_2 was then added to a final concentration of 5 mM and the reaction was cooled at room temperature for 15 min. The annealed RNA was then placed on ice, if to be used immediately, or stored at -20°C until needed. Reactions can be stored at -20°C with no loss of annealing for up to 2 weeks as long as the reaction is thawed at room temperature and then immediately placed on ice when in use. This protocol yields 80–95% annealed RNA, depending on the identity of the RNAs.

Helicase assays

Single turnover helicase assays were performed at 25°C under the following conditions: 1 nM (5.4×10^{-3} μ Ci) bimolecular RNA helicase substrate, 2 mM ATP- Mg^{2+} , 5 mM MgCl_2 , 100 μ M DTT, 0.1 mg/ml bovine serum albumin (BSA), 70 μ M poly(A), 50 mM HEPES pH 7.5, 50 mM KCl and 5% glycerol in a final volume of 10 μ l. DbpA was added last over a range of concentrations (0.1–600 nM). After 30 min, 8 μ l aliquots were loaded directly onto a running non-denaturing 5% polyacrylamide gel (29:1 acrylamide:bis-acrylamide) in 1/3 $\times$ Tris-borate-EDTA (1 $\times$ TBE: 0.9 M Tris-borate, 2 mM EDTA). No quench was added to these reactions since the negatively charged RNA substrate and positively charged protein migrate towards opposite electrodes when loaded into the gel matrix, preventing the reaction from proceeding in the gel. Gels were dried and then quantified with a phosphorimager (Molecular Dynamics Storm 820). The fraction of RNA substrate unwound was calculated as follows: $F(\text{dis})/[F(\text{an}) + F(\text{dis})]$, where $F(\text{dis})$ is equal to the amount of ^{32}P -labeled oligonucleotide that has been displaced from the bimolecular substrate, and $F(\text{an})$ is the amount of ^{32}P -labeled oligonucleotide that is in the annealed complex.

In the case of time course helicase assays, all reactions were performed as above except each reaction contained 300 nM DbpA and was incubated for a specific length of time and stopped by the addition of 9 μ l of the reaction to 3 μ l of 4 $\times$ quench (30 mM EDTA and 0.6% SDS, final concentrations) that denatures the protein but does not disrupt the RNA helix. Aliquots (9 μ l) of the quenched reactions were loaded onto a running, non-denaturing 10% polyacrylamide gel (29:1) in 1/3 $\times$ TBE and dried and quantified as above.

Multiple turnover helicase assays were performed at 10 nM DbpA, 200 nM ^{32}P -labeled (0.54 μ Ci) bimolecular RNA helicase substrate, 5 μ M short oligonucleotide (to serve as a trap for the R32 component of the bimolecular helicase substrate), 2 mM ATP- Mg^{2+} , 5 mM MgCl_2 , 100 μ M DTT, 0.1 mg/ml BSA, 50 mM HEPES pH 7.5, 50 mM KCl and 5% glycerol in a final volume of 10 μ l for each time point. The assays were performed at concentrations well above the K_D to ensure that all of the DbpA was bound to the RNA during the reaction. All reactions were performed at 25°C . Aliquots (9 μ l) were taken at each time point, added to 3 μ l of 4 $\times$ quench (final concentrations: 30 mM EDTA, 0.6% SDS), loaded onto a running, non-denaturing 10% polyacrylamide gel (29:1) in 1/3 $\times$ TBE and dried and quantified as above.

All graphs presented represent the average of at least three data sets.

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