YxiN Is a Modular Protein Combining a DExD/H Core and a Specific RNA-binding Domain*

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DExD/H proteins, typically described as RNA helicases, participate in rearrangement of RNA-RNA and possibly RNA-protein complexes in the cell. Aside from the conserved DExD/H core, members of this protein family often contain N- and C-terminal extensions that are responsible for additional functions. The Bacillus subtilis DExD/H-box protein YxiN and its Escherichia coli ortholog DbpA contain an ~80 amino acid C-terminal extension that has been proposed to specifically interact with a region of 23 S rRNA including hairpin 92. In this study, the DExD/H-box core and the C-terminal domain of YxiN were expressed and characterized as separate proteins. The isolated DExD/H-box core, YxCat, had weak, nonspecific RNA binding activity and showed RNA-stimulated ATPase activity with a $K_m$ (ATP) that resembled several non-specific DExD/H proteins. The isolated C-terminal domain, YxRBD, bound RNA with the high affinity and specificity seen with full-length YxiN. Thus, YxiN is a modular protein combining the activities of the YxCat and YxRBD domains. Footprinting of YxiN and YxRBD on a 172-nucleotide fragment of 23 S rRNA was used to identify the sites of interaction of the C-terminal and helicase domains with the RNA.

Members of the DExD/H helicase family participate in many cellular processes involving rearrangement of RNA-RNA and RNA-protein interactions (1, 2). DExD/H proteins contain a conserved catalytic core consisting of two RecA-like domains that bind ATP and single-stranded RNA (3). Similar to other RecA-like ATPases (4), the cycle of ATP binding, hydrolysis, and release is coupled to a conformational change in the core. In DExD/H proteins, this results in translocation of the protein along single-stranded RNA and subsequent duplex destabilization and/or ribonucleoprotein disruption (5, 6). Outside of the catalytic core, DExD/H proteins often contain ancillary N- and C-terminal extensions that correlate with their specific cellular functions. In many cases, helicases are part of multiprotein complexes, and the putative role of the ancillary domains is to recruit the helicase to the complex by protein-protein interactions (7). Alternatively, the ancillary domains can directly bind to a specific RNA substrate, delivering the helicase to its site of action.

Bacillus subtilis DExD/H-protein YxiN and Escherichia coli DbpA define a group of bacterial orthologs with a conserved C-terminal domain of ~80 amino acids (Fig. 1A). Both YxiN and DbpA specifically recognize the A-site region of 23 S rRNA, including hairpin 92, suggesting that the RNA specificity is conferred by the C-terminal domain (8–11). In a domain swap experiment, the C-terminal domain of YxiN was appended to the catalytic core of SrmB, a non-sequence-specific RNA helicase from E. coli (12). The resulting chimera possessed the RNA specificity of YxiN but had catalytic properties similar to those of the parental helicase, SrmB. These results indicate that the C-terminal domain of YxiN is capable of imparting specificity in the context of another DExD/H box protein, suggesting that the sequence-specific RNA binding and helicase activities are separable and that this group of DExD/H proteins may be functionally modular. To prove this hypothesis, the helicase and C-terminal domains of YxiN were prepared here as separate polypeptides. This approach permits the intrinsic activities of the catalytic core and the C-terminal domain to be assayed independently and compared with the full-length protein.

**EXPERIMENTAL PROCEDURES**

**Plasmids for Expression of Recombinant YxiN Fragments—**Coding sequences for YxiN protein fragments were PCR-amplified from the native B. subtilis sequence in an existing plasmid (11) and cloned by restriction/ligation into the pTWIN1 vector of an intein-based expression system (New England Biolabs) using the unique NdeI and SpeI sites of the vector. Primers were designed such that the subcloned fragments deleted the first of the tandem inteins of the parent vector and placed the C-terminal residue of each encoded YxiN protein fragment flush with the N-terminal residue of the second intein. In this manner, no extraneous residues remained at the C terminus of the target after self-cleavage of the target-intein fusion. The plasmids used for these studies encoded the DExD/H core fragment, termed YxCat (residues 1–368) and the C-terminal domain fragment, termed YxRBD (residues 404–479, which starts at Met-404 of YxiN).

**Protein Expression and Purification—**Full-length YxiN from a previous study was used (11). YxiN fragments were expressed in E. coli BL21(DE3) either by growing in LB to $A_{600}$ of ~0.6 and inducing with 0.4 mM isopropyl 1-thio-β-D-galactopyranoside or by growing in a self-inducing medium (13). Cells were harvested, suspended in 20 mM Tris-HCl, 500 mM NaCl, pH 7.9, at 20 °C (Buffer A) and lysed by sonication. Protein purification was carried out at 4 °C. After removal of cell debris by centrifugation, the clarified supernatant was brought to 0.1% (w/v) in polyethyleneimine by dropwise addition from a 10% (w/v) stock solution, pH 7.2, over a period of 10 min. Precipitate was removed by centrifugation, and the supernatant was applied to a chitin column and washed with several column volumes of Buffer A. Then, the column was loaded with 50 mM dithiothreitol in Buffer A, and flow was stopped overnight to allow on-column self-cleavage of the target-intein fusion. The next day, protein was eluted from the column in 2 column volumes of wash, concentrated to a volume of a few milliliters, and loaded onto a gel filtration column (Superdex-200 (Amersham Biosciences) for fragment 1–368; Superdex-75 for fragment 404–479) pre-equilibrated with 20 mM Tris-HCl, 100 mM NaCl, pH 7.9, at 20 °C (Buffer B). Protein from the peaks corresponding to the YxiN fragments were concentrated to >5 mg/ml and stored at −70 °C in Buffer B.

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**YxiN is a Modular DExD/H-box Protein**

RNA Substrates—RNAs HP +15 M and HP +15 through HP +2 were purchased from Dharmaco, Inc. Poly(A) RNA was purchased from Roche Diagnostics. 172-nt RNA was prepared by run-off transcription as described previously (10).

**Gel Mobility Shift Binding Assay**—The binding assay was based on the previously described method (14). A low concentration (0.4 nM) of 5'32P-labeled RNA was mixed in 16-μl reactions with a series of protein concentrations in 50 mM HEPEs, pH 7.5, 50 mM KCl, 5 mM MgCl2, 100 mM dithiothreitol, 70 mM poly(A), 5% (v/v) glycerol, and 0.1% (v/v) Tween 20. Poly(A) was omitted from the YxCat binding experiments. Reactions were incubated for 10 min to reach equilibrium, and 10-μl aliquots were loaded onto 5% native acrylamide gels (29.1 ratio of acrylamide to bis-acrylamide) in 0.33× TBE (1× TBE is 90 mM Tris borate, 1 mM EDTA, pH 8.3). Gels were run in 0.33× TBE at 200 V, dried, and exposed to a phosphorimaging screen. The fraction of unbound material was quantitated as the ratio of counts in the free RNA band to the combined counts of the free RNA and the shifted species above. The fraction of bound material (1 − unbound fraction) was fit to a noncooperative binding curve to derive the dissociation constant. Reported ΔG and Kd values resulted from averages of three experiments. Typical standard deviations of ΔG values were below 0.7 kcal/mol (−3-fold in Kd), and individual standard deviations are depicted as error bars in Fig. 3.

**Footprinting Assay**—172-nt RNA substrate footprinting using RNAses T1, T2, and kethoxal was carried out as described previously (10). Where noted, 1 μM His-DpbA, 1 μM YxiN, 1 μM YXRBD, and 5 μM AMPPNP-Mg were used.

**ATPase Assay**—The rate of ATP hydrolysis was measured using the previously described coupled spectrophotometric assay (9) at room temperature with the addition of 0.1% (v/v) Tween 20. The individual cuvette method was modified to a high-throughput plate format using the SpectraMax Plus 384 spectrophotometer (Molecular Devices). In a single experiment, duplicate 50-μl reactions were placed in flat-bottom polystyrene 384-well plates, and absorbance was monitored at 338 nm for 15 min. Individual well path lengths experimentally determined by the instrument (PathCheck) were used to normalize the absorbances to a path length of 1 cm. Data from at least two independent experimental measurements were used in fitting. Typical standard deviations of the reported ATP hydrolysis rates at or above half-maximal ATPase stimulation were below 10%, and individual standard deviations are depicted as error bars in Fig. 6. RNA titration experiments used 200 nM YxiN and 1 μM YxCat. In the Kd(15N) determination, 50 μM poly(A) and 200 nM YxiN and YxCat were used. The fully general kinetic equation was used when the concentration of enzyme was near the Kapp(15N) (11).

**RESULTS**

**Gel Mobility Shift Binding of RNA by YxiN, YxRBD, and YxCat**—The RNA binding properties of *B. subtilis* YxiN are similar to those of its *E. coli* ortholog DpbA. Previous experiments demonstrated a specific interaction of YxiN with RNAs containing hairpin 92 of 23 s rRNA, such as HP +15 (Fig. 1B), by an RNA-stimulated ATPase assay, whereas mutation of two bases in the stem loop of the RNA (RNA HP +15M) eliminated the stimulation (12). To directly examine the binding and specificity of YxiN, a gel-shift assay was used. Trace concentrations of 5'32P-labeled RNA HP +15 were mixed with varying concentrations of YxiN and analyzed on a native gel. A distinct, slower migrating band was observed (Fig. 2A) consistent with complex formation between RNA HP +15 and YxiN. The smear below the complex is considered to represent specific complex that falls apart during the electrophoresis and is counted as the bound fraction in quantitation. The plot of fraction bound versus protein concentration fits a sigmoid binding curve with Kd of 4.6 nM (Fig. 4). The curve has a Hill coefficient of ~1, indicating noncooperative binding of HP +15 with YxiN. In a similar experiment using the nonspecific RNA HP +15M (Fig. 2B), no complex band was observed even at 5 μM YxiN, a 10-fold higher concentration than the highest used with RNA HP +15. Thus, base mutations in the loop of RNA HP +15 disrupt its interaction with YxiN, indicating that the binding is sequence-specific. DpbA has shown an analogously specific interaction with RNA HP +15 by stimulation of ATPase activity (15) and a similar Kd in electrophoretic mobility shifts (9.2 nM) (14).

The interaction between YxiN and its specific RNAs has been proposed to involve sequence specific binding of the C-terminal domain and sequence nonspecific binding of the catalytic core (12). To test this proposal directly, the two portions of the gene were cloned separately as fusions with an intein and a chitin-binding domain (pTWIN1, New England Biolabs). YxCat, the fragment of YxiN containing the catalytic core, extends from residues 1–368, including 35 amino acids after the last highly conserved motif (H/Q)RAXGRXRGR (Fig. 1A). This site was chosen because it aligns close to the end of the minimal DExD/H protein eIF4A. YXRBD, the C-terminal fragment of YxiN, encompasses a region of conserved sequence between residues 404–479. YxiN and YxRBD were not included because this region varies in length among DpbA orthologs (7–35 amino acids) and may represent a flexible linker. Expression of these fragments yielded large amounts of soluble proteins that were purified and cleaved on a chitin column and further purified by gel filtration.15N,1H-HSQC NMR spectra of 15N-labeled YXRBD revealed a dispersion of amide proton peaks that is consistent with a folded polypeptide. In addition, crystals of the fragment have been obtained, indicating that it constitutes a well folded domain (data not shown).

The individually expressed fragments of YxiN both show RNA binding activity. The addition of the C-terminal fragment, YXRBD, to RNA HP +15 shows a shifted band corresponding to the RNA-protein complex (Fig. 2C). As would be expected for its smaller size, the mobility shift by this protein fragment is less than that of full-length YxiN. The affinity of YXRBD to RNA HP +15 (5.2 nM) is similar to that of YxiN (4.6 nM) (Fig. 4). Furthermore, interaction of YXRBD with RNA HP +15 is not observed, paralleling the sequence specificity of full-length YxiN (Fig. 2D). The catalytic core fragment, YxCat, interacts with RNA HP +15 to produce a faint complex band of intermediate mobility at micromolar protein concentrations (Fig. 2E). The binding is not saturated even at the highest concentrations of YxCat tested, indicating a very weak interaction (Kd > 4 μM). YxCat binds RNA HP +15 just as well as it does RNA HP +15 (Fig. 2F), demonstrating that the catalytic core fragment does not have RNA sequence specificity. Thus, YXRBD interacts with RNA tightly and sequence specifically, whereas YxCat binding to RNA is weak and is not sequence-specific.

It was of interest to determine the minimal length of the 5' single-stranded extension in RNA HP +15 that is needed to bind YxiN and YXRBD. To this end, a set of RNAs with single nucleotide truncations from the 5' end of RNA HP +15 (Fig. 1B) were each used in gel-shift binding experiments with YxiN and YXRBD. The resulting free energies of binding are shown in Fig. 3. Surprisingly, neither YxiN nor YXRBD showed a sharp binding “boundary” where the affinity drops dramatically within one or two nucleotides. Instead, the energies of binding to both proteins decreased gradually with decreasing 5' extension length. For RNAs with more than seven nucleotides of 5' single-stranded...
sequence, binding affinity to YxRBD is the same as to YxiN. However, shorter RNAs show differences in their binding behavior to YxRBD and YxiN (Fig. 3). For the shortest RNAs, HP+2 and HP+3 (Fig. 1B), binding to full-length YxiN can still be observed, whereas neither RNA shows detectable binding to YxRBD (Fig. 4). The disparity in binding properties of the two proteins suggests that the catalytic domains of YxiN, which are absent in YxRBD, may contribute to the binding of shorter RNAs. However, the addition of more single-stranded residues to the short RNAs increases their binding affinity to both YxRBD and YxiN (Fig. 3). Because the longer RNAs bind more tightly than short RNAs to YxRBD (which lacks the catalytic domains), in this case the additional binding energy likely arises from nonspecific electrostatic interactions of the single-stranded region with the basic C-terminal domain. Thus, because nonspecific interactions of the RNA can occur both with the C-terminal and the catalytic domains, the resulting affinities of YxRBD and YxiN for longer RNAs become similar to each other.

Footprinting of YxiN and YxRBD on RNA—DbpA and YxiN have been shown to interact with a larger region of 23 S rRNA than RNA HP+15. Although the hairpin of RNA HP+15 (hairpin 92 in 23 S rRNA) is a major affinity and specificity determinant, DbpA and YxiN show increased binding affinity and ATPase stimulation with larger RNAs that also contain helices 89, 90, and 93 in addition to 92 (14, 15). DbpA has an extensive footprint on a 172-nt RNA that includes this region (Fig. 5), indicating the presence of additional contacts of DbpA with RNA elements other than hairpin 92 (10).

The YxRBD fragment is useful in assigning the domains of YxiN to specific interactions with such larger RNAs because it can identify the site of binding of the C-terminal domain devoid of the catalytic core. To this end, the footprint of YxRBD on the 172-mer was obtained using RNases T1, T2, and kethoxal and was compared with analogous footprints of YxiN in the presence of AMPPNP. In addition, the footprint of DbpA with AMPPNP on the same RNA was used as a control. The results of the footprinting experiments are shown on the secondary structure of the 172-mer in Fig. 5. All three proteins showed protections from kethoxal at G2553 in hairpin 92, and from RNase T2 at U2652/A2564 in the bulge between helices 92 and 90. Protection at G2553 was also confirmed by RNase T1 (data not shown). However, only the full-length DbpA and YxiN protected the G2502/G2505 residues between helices 89 and 90, and the G2588/G2603 residues in helix 93 from RNase T1 and kethoxal (Fig. 5 and data not shown). Footprinting in the bulge of

FIGURE 1. A, domain structure of YxiN and a sequence alignment of its C-terminal region. Rectangles show the two RecA-like domains of the DExD/H core and the presumed C-terminal domain. Roman numerals denote the conserved motifs in the DExD/H core. Arrows in the alignment indicate amino acid positions in YxiN. The alignment was generated using ClustalW and is shaded at 70% similarity. B, RNAs used in the current study.
elicits full activation of 55 min\(^{-1}\) at the lowest RNA concentration in this experiment (200 nM). Using a lower range of RNA concentrations, the apparent activation constant of RNA HP + 15 (\(K_{\text{app}}\)) was determined to be 31 nM (TABLE ONE). In contrast, stimulation by RNA HP + 15 M occurs with a much higher \(K_{\text{app}}\) of 3500 nM and saturates at a lower maximal activity (25 min\(^{-1}\)). Poly(A) also activates YxiN weakly, increasing activity linearly up to a concentration of 10 \(\mu\)M without reaching saturation (the concentration of poly(A) is expressed in 32-nt units). To gauge the effectiveness of such non-saturating substrates, the apparent second order rate constant (\(k_{\text{app}}/K_{\text{app}}\) (RNA)) was derived from the initial slope of ATPase activity versus RNA concentration (TABLE ONE). Under these conditions, YxiN exhibits a 250-fold specificity for RNA HP + 15 over RNA HP + 15 M and a 1000-fold specificity over poly(A). Previous experiments with YxiN at the more stringent salt concentration of 150 mM showed a specificity of 170,000 for RNA HP + 15 over RNA HP + 15 M (12).

The isolated catalytic core maintains the capacity for RNA-stimulated ATPase activity (Fig. 6B). Similar to full-length YxiN, the RNA-independent ATP hydrolysis rate of YxCat was found to be 0.40 min\(^{-1}\) (data not shown). Upon addition to 1 \(\mu\)M YxCat, RNAs HP + 15, HP + 15 M, and poly(A) stimulate the ATPase activity linearly with RNA concentration and show similar second order rate constants between 5.4 \times 10^{-5} \text{ and } 6.9 \times 10^{-5} \text{ min}^{-1} \text{ nM}^{-1} \text{ (Fig. 6B and TABLE ONE). The lack of saturation up to 10 \(\mu\)M RNA concentrations may reflect the more acidic pH of YxCat (5.8 compared with 7.7 for YxiN), making it less likely to interact with RNA at the assay pH of 7.5. In fact, titrations with poly(A) remain linear up to 50 \(\mu\)M (in 32-nt units), eliciting rates up to 3.5 min\(^{-1}\) (data not shown). Because saturation of ATPase activity was not attained, the \(k_{\text{max}}\) of YxCat can only be estimated to be between 3.5 min\(^{-1}\) and the full-length YxiN value of 55 min\(^{-1}\). Assuming this range of \(k_{\text{max}}\), the calculated apparent RNA binding affinities (\(K_{\text{app}}\)) for RNAs in TABLE ONE are in the range of 65 to 1000 mM. Thus, YxCat has very weak apparent RNA binding affinity and no intrinsic specificity among the tested RNAs. Finally, because the C-terminal fragment does not contain any ATPase motifs, it does not hydrolyze ATP on its own, as expected (data not shown).

Two experiments suggest that the ATPase activity of YxCat is functionally independent from the C-terminal fragment. First, the apparent affinity of YxCat for ATP was compared with that of YxiN. The ATPase activity of both proteins was assayed at varying concentrations of ATP-Mg with 50 \(\mu\)M poly(A) (in 32-nt units) (Fig. 6, C and D). The resulting \(K_m\) values of 210 \(\mu\)M for YxiN and 350 \(\mu\)M for YxCat are similar to each other and close to the value of 230 \(\mu\)M measured with saturating
amounts of the specific 23 S + 16 S rRNA substrates (11). Thus, YxCat does not require the C-terminal fragment for proper formation of the ATP site and for ATP binding. Second, when 1 μM YxRBD was added to the YxCat ATPase assay with either RNA HP1 or HP15, no additional stimulation or preference for RNA HP15 was observed (data not shown). This indicates that YxRBD is incapable of selectively presenting the specific RNA to YxCat in trans, suggesting a lack of interaction between the catalytic core and the C-terminal domain.

DISCUSSION

The present study demonstrates that the C-terminal region of YxiN is a domain that can independently bind RNA in a sequence-specific manner. Expressed as a 76-amino acid fragment, YxRBD folds well and specifically binds RNAs containing hairpin 92 of 23 S rRNA with low nanomolar affinity (Fig. 2C and D). This domain is similar in size to the currently known RNA-binding domains of the RRM (RNA recognition motif), dsRBD (double-stranded RBD), and OB (oligonucleotide/oligosaccharide binding)-fold families, as well as to the small, single-domain ribosomal proteins. Although assignment to the OB-fold family cannot be made based on sequence (16), alignments of YxiN, DbpA and other bacterial homologs (Fig. 1A) indicate that the C-terminal domain defines a distinct subfamily of RNA-binding domains. However, in a Pfam search, a few members of the subfamily show very weak similarity with the eukaryotic RRM family (expectation values, >0.05) in a 20-amino acid region starting at RNP-1 (17). A closer examination of a possible alignment indicates that half of the six most conserved positions of the RRM (Leu-16, Val-38, and Ala-49, numbering by Birney et al. (18)) have analogous positions in the DbpA C-terminal domain, whereas the other half does not (Leu-7, Phe-20, and Phe-40) (18). Thus, the precise classification of this sequence specific RNA-binding domain awaits further studies.

Aside from orthologs of DbpA, there is a highly similar, but distinct, C-terminal domain present in E. coli DeaD/CsdA, a DExD/H protein involved in the ribosomal biogenesis of the 50 S subunit (19). In CsdA and its orthologs, the domain is located significantly further away from the helicase core and is flanked on both sides by arginine- and glycine-rich sequences that are known to accompany other RNA-binding domains (20). The C-terminal domain in CsdA homologs differs from the YxiN/DbpA RBD in several conserved sequence positions (the two groups are defined together in Pfam entry PF03880). Interestingly, CsdA does not exhibit any detectable in vitro specificity for hairpin 92, and its ATPase activity is stimulated by several RNAs (21). These results suggest that CsdA either lacks RNA specificity or recognizes a relatively common RNA motif.

Characterization of the YxCat fragment indicates that it possesses the
essential catalytic properties shared by all DExD/H proteins. The \( K_m \) for ATP is 350 \( \mu M \) for YxCat and 210 \( \mu M \) for YxiN, which are similar to the values of 330 \( \mu M \) reported for eIF4A (22), 260 \( \mu M \) for Prp16 (23), 180 \( \mu M \) for SrmB (12), and 300 \( \mu M \) for Ded1p (24). The similarity in the ATP binding properties of YxCat with the intact protein, as well as with the other DExD/H proteins, suggests that the ATP pocket is properly formed within the isolated catalytic core. YxCat binds RNA very weakly, with a \( K_d \) greater than 4 \( \mu M \) and a \( K_{app}(RNA) \) greater than 65 \( \mu M \). Similarly, the minimal DExD/H-box protein eIF4A binds poly(A) with a \( K_{app}(RNA) \) of 115 \( \mu M \) (in 20-mer units) (22). However, ancillary domains present in other DExD/H proteins may aid in the interaction with RNA, increasing the affinity. For example, the yeast DExD/H protein Ded1p binds RNA with a \( K_d \) of 20 \( nM \) (24), and the hepatitis C virus, NS3, has a 2 \( \mu M \) \( K_{app}(RNA) \) (25). Thus, tight intrinsic RNA affinity of the helicase cores does not appear to be required generally, presumably because of their function in larger polypeptide chains or complexes. As in most DExD/H proteins, RNA binding to YxCat stimulates ATP hydrolysis (Fig. 6B) with rates comparable with those of eIF4A (3 min\(^{-1}\)). Furthermore, both RNA binding and stimulation of ATPase activity of YxCat are not sequence specific. Likewise, most of the DExD/H proteins studied in vitro lack sequence specificity, suggesting that this is a general property of DExD/H cores. Consistent with this notion, DExD/H helicases have been proposed to interact primarily with the oligonucleotide backbone (26).

The individual domains of YxiN appear to function in a modular fashion. The C-terminal domain provides the bulk of the binding energy as well as sequence specificity, whereas the helicase core performs the catalytic functions. The two domains do not require each other for activity, and there appears to be no interaction between them, because the addition of YxRBD to YxCat in trans does not increase the ATPase activity or impart specificity. Thus, the C-terminal domain simply brings the catalytic core to its cellular target. Such a modular design strategy may be common for DExD/H proteins with ancillary domains. For example, modularity in the yeast splicing DExD/H protein Prp16 was established by testing domain deletion mutants of Prp16 for viability in trans.

**TABLE ONE**

| RNA-dependent ATPase activity constants of YxiN and YxCat |
|----------------------------------------------------------|
| **Protein/RNA** | **k\(_{max}\)** | **K\(_{app}\)** | **k\(_{max}\)/K\(_{app}\)** | **Specificity**^a |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| YxiN            |                 |                 |                 |                 |
| RNA HP +15      | 55              | 31              | 1.8             | 1               |
| RNA HP +15 M    | 25              | 3500            | 7.1 \times 10^{-3}| 0.004           |
| Poly(A)         |                 |                 | 1.7 \times 10^{-3}| 0.001           |
| YxCat           |                 |                 |                 |                 |
| RNA HP +15      | 5.4 \times 10^{-5}| 1               |
| RNA HP +15 M    | 6.9 \times 10^{-5}| 1.3             |
| Poly(A)         | 6.9 \times 10^{-5}| 1.3             |

^a Specificity is defined as the ratio of second order rate constants: \( k_{max}/K_{app}(RNA)/k_{max}/K_{app}(RNA \ HP +15) \).
vivo and interactions with the spliceosome in vitro (27). The N-terminal ancillary domain was found to interact with the spliceosome and act as a dominant negative inhibitor of splicing in vitro. Thus, the N-terminal domain functions to bring the protein to the spliceosome. However, in contrast with YxiN, the N-terminal domain of Prp16 in trans with the other domains of Prp16 can rescue a strain devoid of Prp16, indicating that the N-terminal domain interacts with the rest of the protein.

The modular nature of YxiN sheds light on how it interacts with its larger, more biologically relevant 172-mer substrate, as well as potential modes of action on the cellular substrate of YxiN. The footprinting pattern of YxRBD indicates that this domain interacts sequence-specifically with hairpin 92 and the single-stranded sequence connecting it to helix 90, tethering the catalytic core to this location (Fig. 5). The absence of RNA specificity of YxCat indicates that the full-length enzyme has the ability to act on any suitable substrate in the vicinity of its molecular localization. Accordingly, within the 172-mer RNA, the catalytic core interacts with regions encompassing positions 2502 and 2505 5′ of helix 90, as well as 2588 and 2603 3′ of helix 90. The flexibility of the catalytic core relative to the tethering point of the C-terminal domain, reflected in the ability of DbpA to unwind duplexes in several locations around helix 90 (28), suggests that YxiN and DbpA may interact with the regions 5′ and 3′ of helix 90 in two distinct binding populations. The function of YxiN and DbpA may be to unwind an RNA duplex or rearrange a ribonucleoprotein structure at one or both of these locations during the ribosomal lifecycle. Recently, DbpA has been shown to unwind duplexes in a 3′-to-5′ direction, regardless of the orientation of the duplex relative to hairpin 92, and to require a 3′ single-stranded loading site, presumably for the binding of the catalytic core (29). A similar requirement of a 3′ single-stranded RNA loading site may exist for its natural targets. In the context of the entire cellular substrate of YxiN, the 50 S ribosomal subunit or its assembly precursor, the catalytic domains of YxiN may act at the same site(s) as in the 172-mer or rearrange a different target or targets near hairpin 92. Rearrangement of several interactions near hairpin 92, an area crucial to ribosomal function, may be required for proper RNA folding and establishment of correct interactions with ribosomal proteins.

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