Kinetics and Thermodynamics of DbpA Protein’s C-Terminal Domain Interaction with RNA

Aliana López de Victoria, Anthony F. T. Moore, Apostolos G. Gittis, and Eda Koculi

INTRODUCTION

DbpA is an *Escherichia coli* DEAD-box RNA helicase implicated in RNA structural isomerization in the peptide bond formation site. Like all of the members of the DEAD-box family of enzymes, DbpA contains a RecA-like catalytic core, which is the workhorse of the protein. The DbpA catalytic core uses the energy of ATP binding and hydrolysis to unwind short double-helix RNA substrates in a sequence nonspecific manner. Structural data obtained with many members of DEAD-box proteins in complex with RNA, and biochemical data obtained with DbpA and its ortholog in *Bacillus subtilis*, YxiN, suggest that the ATP binding to the DbpA catalytic core promotes its loading onto one of the strands of the double-helix substrate. Next, ATP is hydrolyzed and the RNA strand, complementary to the double-helix strand to which the DbpA catalytic core was loaded on, is released. Inorganic phosphate release produces the release of the second RNA strand. Finally, ADP release regenerates the enzyme. Similar to many other members of the DEAD-box family of enzymes, DbpA hydrolyses one ATP molecule for every short double-helix unwinding event. Moreover, the interaction of a single-stranded RNA region with the catalytic core of DbpA is sufficient to stimulate DbpA ATPase activity. In addition to the catalytic core, DbpA contains a structured C-terminal domain. This domain is an RNA recognition motif (RRM), and it imparts specific and tight binding of DbpA to hairpin 92 of 23S ribosomal RNA. The function of the RNA-binding domain of YxiN/DbpA and its catalytic core are completely autonomous. More importantly, our recent study demonstrates that the DbpA catalytic core could unwind any double-helix substrate within its grasp, suggesting that during ribosome assembly, once the DbpA protein is bound to hairpin 92 via its RNA-binding domain, the catalytic core could unwind double-helices placed at different regions of the ribosomal particle. Experiments performed with the helicase inactive DbpA construct, R331A, seem to support this hypothesis.

When the helicase inactive DbpA construct, R331A, is expressed in *E. coli* cells, three large subunit intermediate particles accumulate with sedimentation coefficients of 27S, 35S, and 45S. The protein composition of the 45S particle is known from the quantitative mass spectrometry. In the 45S particle, six late-stage ribosomal subunit proteins are less abundant and one is completely missing. The less abundant proteins are positioned in different regions of the large ribosomal subunit, suggesting that DbpA may perform RNA structural rearrangement in more than one area of the ribosomal particle. The modeling data of DbpA structural isomerization in the peptide bond formation site implicated in RNA structural rearrangement in the peptide bond formation site.

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INTRODUCTION

DbpA is an *Escherichia coli* DEAD-box RNA helicase implicated in RNA structural isomerization in the peptide bond formation site. Like all of the members of the DEAD-box family, DbpA contains a structured C-terminal domain, which is responsible for anchoring DbpA to hairpin 92 of 23S ribosomal RNA. The function of the RNA-binding domain of YxiN/DbpA and its catalytic core

DbpA could unwind a number of double-helix substrates before its dissociation from the ribosomal particles.

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bind to hairpin 92 via its C-terminal RNA-binding domain and unwinds a number of double-helices before the C-terminal domain dissociates from the ribosomal RNA? The experiments outlined here aim to determine the residence time of the DbpA C-terminal domain on RNA and compare it to the time required by the DbpA catalytic core to hydrolyze one ATP molecule and release ADP. The knowledge of the residence time of DbpA on RNA is important for a complete understanding of the mechanism DbpA employs to perform RNA structural rearrangements during ribosome assembly.

**RESULTS**

**Kinetics of DbpA Binding to RNA.** The goal of these experiments was to determine the kinetics of association and dissociation of DbpA to the cognate RNA shown in Figure 1A. Model molecules similar to the one shown in Figure 1A, have been used by our laboratory and other laboratories to measure the DbpA–RNA interaction. Extensive amounts of previous data from other laboratories have shown that in the absence of ATP, the C-terminal RNA-binding domain of DbpA is the only domain of DbpA interacting with RNA. Thus, under our experimental conditions, in the absence of ATP, we are measuring the association and dissociation of the DbpA C-terminal domain to RNA. Surface plasmon resonance (SPR) was employed to measure the kinetics of association and dissociation of DbpA to the RNA molecule shown in Figure 1A. Figure 1B shows the model RNA molecule used in the reference flow cell of the SPR experiment. DbpA–RNA association was monitored for 180 s, whereas DbpA–RNA dissociation was monitored for 360 s.

![Figure 1. SPR kinetic data. (A) RNA molecule immobilized on the sample cell. The RNA molecule immobilized on the sample cell contains hairpin and helix 92 of the 23S rRNA. Hairpin and helix 92 are required for tight and specific binding of DbpA to RNA. (B) RNA molecule immobilized on the reference cell. (C) Representative adjusted sensograms of nine different protein concentrations showing kinetics of association and dissociation of the DbpA protein to the 42-mer RNA (A). The adjusted sensograms were obtained by subtracting the signal obtained from the experimental cell from the signal obtained from the reference cell. The continuous lines represent global fits of the data collected at the given protein concentration. DbpA–RNA association was monitored for 180 s, whereas DbpA–RNA dissociation was monitored for 360 s.](image-url)
signal is proportional to the change in the molecular mass of the ligand–analyte complex. Consequently, the change in signal is expected to be much larger when the lower-molecular-mass 42-mer RNA (14.5 kDa) is attached to the chip surface and the higher-molecular-mass DbpA (55 kDa) serves as the analyte.

Figure 1C is an example of the differences in adjusted sensograms obtained at various DbpA concentrations in the presence of 50 mM KCl. The sensograms consist of three phases: the association phase, during which DbpA is flowed across the chip and there is an increase in the response units over time as the DbpA–RNA complex forms; the equilibrium or steady-state phase, during which the response units do not change; and the dissociation phase, during which the DbpA is removed from the flow cell.

By performing a mathematical global fit of the SPR data and using the 1:1 binding model (Materials and Methods, eqs 1–4) we obtained the microscopic rate constant of association (kₐ), the microscopic rate constant of dissociation (kₐ), and equilibrium dissociation constant (K_D) of DbpA cognate RNA interaction at 50 mM KCl. From these fits, the kₐ = (1.4 ± 0.2) × 10⁸ M⁻¹ s⁻¹, and the kₐ = (8.0 ± 0.4) × 10⁻⁸ s⁻¹. The K_D obtained from the ratio of kₐ to kₐ is 56.9 ± 0.2 nM, whereas the K_D obtained from the steady-state binding levels dependence on the DbpA concentrations is 60.3 ± 0.4 nM. Hence, the K_D values obtained from the ratios of the microscopic rate constants and the equilibrium, steady-state binding levels are similar.

The detection limit for kₐ measurement with the Biacore X100 is 1.0 × 10⁷ M⁻¹ s⁻¹, whereas the detection limit for kₐ measurement is 0.1 s⁻¹. At KCl concentrations lower than 50 mM, kₐ increases to values higher than 1.0 × 10⁷ M⁻¹ s⁻¹, and at KCl concentrations higher than 50 mM, the kₐ increases to values larger than 0.1 s⁻¹. Hence, we were only able to accurately measure kₐ and kₐ using a KCl concentration of 50 mM. The increase in kₐ values observed at KCl concentrations lower than 50 mM suggests that long-range electrostatic interactions drive the recognition of RNA by DbpA. The lower monovalent salt concentration promotes a faster RNA–DbpA complex formation. The increase in kₐ for KCl concentrations higher than 50 mM KCl suggest that electrostatic interactions, which become weaker at higher monovalent concentration, play a role in the stability of the DbpA–RNA complex. Thus, in addition to facilitating the recognition of RNA by DbpA, electrostatic interactions may also promote the stability of the DbpA–RNA complex. The role of electrostatic interactions in promoting both the recognition of the RNA by protein and the stability of protein–RNA complex has been previously demonstrated for the N-terminal domain of U1A splicingosomal protein interaction with its cognate RNA. Similar to DbpA’s C-terminal domain, the N-terminal domain of U1A is an RRM.

Kinetics and Equilibrium of ATPase Activity of DbpA. The activation of ATP hydrolysis by DbpA in the presence of various ATP concentrations and 2 μM of RNA was measured by using the pyruvate kinase/lactate dehydrogenase-coupled assay as explained in Materials and Methods section of the article. The RNA molecule used in this experiment is shown in Figure 1A. The pyruvate kinase lactate/dehydrogenase-coupled assay measures the amount of ADP formed as a consequence of ATP hydrolysis by DbpA. Figure 2 shows the dependence of the ATP hydrolyzed by DbpA on the ATP concentration. The Michaelis–Menten equation was used to fit the data in Figure 2. From the data fit, the Michaelis–Menten binding constant (K_m) and the ATP turnover number (k_cat) were obtained. The average values and standard deviation for K_m and k_cat calculated from four independent experiments are shown in Table 1.

Figure 2. Activation of DbpA’s ATPase activity. The RNA molecule used for these experiments is the molecule shown in Figure 1A. The Michaelis–Menten formalism was used to fit the data. The program used to fit the data is KaleidaGraph. The data are representative of a single independent experiment. From these data, the ATP Michaelis–Menten binding constant (K_m) and the ATP turnover number (k_cat) were obtained. The average values and standard deviation for K_m and k_cat calculated from four independent experiments are shown in Table 1.

DISCUSSION

The experiments carried out in this work investigate the microscopic rate constant of association and dissociation of the DbpA RNA-binding domain to its cognate RNA. The microscopic rate constant of the DbpA RNA-binding domain dissociating from its cognate RNA is tenfold lower than the observed microscopic rate constant of ATP hydrolysis by the DbpA catalytic core and release of the ADP molecule. The ADP release is the final step of the DbpA catalytic cycle. Hence, these data combined with our previous studies on region specificity of DbpA and De La Cruz’s group studies, which demonstrated that the hydrolysis of one ATP molecule by DbpA is sufficient to unwind a short double-helix, suggest that when the DbpA protein binds to hairpin 92 during ribosome assembly, it could unwind many double-helices in various regions of the ribosomal particle before it dissociates from this hairpin.

In vitro experiments with a model substrate have shown that not every DbpA ATP hydrolysis cycle produces an unwinding event; in other words, DbpA ATP futile cycles are observed. The number of DbpA’s futile ATP cycles increases with an
increase in the number of bases in the double-helix, which suggests that the futile ATP cycling is a consequence of the reannealing of partially unwound RNA double-helices. The long residence time of the C-terminal RNA-binding domain on the ribosomal particle could also give the catalytic core a chance to unwind the double-helices that the catalytic core was unable to unwind during its futile ATP cycles.

Anisotropy experiments performed by De La Cruz’s group showed the residence time of DbpA on the RNA strand containing hairpin 92 was longer than the time required for the DbpA catalytic core to unwind a short RNA helix. Hence, our data agree with De La Cruz’s group’s conclusions.

Klostermeier’s group demonstrated that the binding of RNA and ATP to YxiN catalytic core is a cooperative process. The single-molecule FRET dyes for these experiments were placed on the catalytic core of YxiN. Thus, these experiments probed only the movement of the two catalytic core RecA-like domains and produced no information on the role of ATP binding on YxiN RNA-binding domain affinity for RNA.

The diagram in Figure 3 describes the interaction of the DbpA RNA-binding domain with RNA and the catalytic cycle of DbpA. Our experiments and experiments performed by Uhlenbeck and McKay groups have demonstrated that the functions of the RNA-binding domain and the catalytic core of DbpA/YxiN are separated. Moreover, Klostermeier and Uhlenbeck groups’ experiments have shown that DbpA/YxiN catalytic core interacts with RNA only in the presence of ATP. Thus, the $k_d$ and $k_a$ measured in the absence of ATP describe the interaction of RNA-binding domain with RNA separated from the interaction of the catalytic core with RNA.

In the presence of AMPNP, the catalytic core of DbpA/YxiN is stuck with RNA in its catalytic jaws. This long residence time of DbpA/YxiN catalytic core is not part of DbpA/YxiN normal catalytic cycle. Hence, we believe that the $k_d$ and $k_a$ measurements in the presence of AMPNP would not produce useful information about DbpA protein’s residence time on RNA. In E. coli, under nonstress conditions, there are 60–150 mM K+ and 2–5 mM of free Mg2+ and 2 mM of polyamines present. Our measurements of $k_d$, $k_a$, and $k_{cat}$ were performed in the presence of 5 mM MgCl2 and 75.9 mM K+ (50 mM from KCl added to the reaction and 25.9 mM K+ from the HEPES–KOH buffer); hence, our experiments were performed within the boundaries of ionic cellular conditions.

### MATERIALS AND METHODS

**Chemicals and RNA Molecules.** All of the chemicals were purchased from Fisher Scientific. The RNA constructs were purchased high-performance liquid chromatography-purified from Integrated DNA Technologies. The streptavidin-coated chips were purchased from GE Healthcare Life Sciences. Pyruvate kinase/lactate dehydrogenase enzyme mixture was purchased from Sigma-Aldrich.

**DbpA Expression and Purification.** DbpA was expressed and purified as previously described. In brief, the DbpA protein bearing a six-histidine N-terminal tag was purified using affinity chromatography followed by gel-filtration chromatography. The histidine tag was not removed from the protein during the purification process.
**Surface Plasmon Resonance.** Figure 1 shows the RNA molecules used for the SPR experiments. Both molecules were chemically synthesized with a biotin molecule at the 5′ end. The DbpA cognate RNA (Figure 1A) was immobilized via the biotin–streptavidin interaction in the sample cell, whereas the linear RNA (Figure 1B) was immobilized via biotin–streptavidin interaction in the reference cell. To ensure that hairpin 92 was correctly formed, DbpA’s cognate RNA was heated to 95 °C in the presence of 50 mM HEPES–KOH pH 7.5 and 50 mM KCl and kept at 95 °C for 1 min. Subsequently, the reaction mixture was cooled down to 65 °C and kept at that temperature for 3 min, and then cooled down again to 22 °C. Next, MgCl₂ was added to a final concentration of 10 mM and the RNA sample was incubated at 22 °C for 15 min. This annealing protocol has been used previously to anneal the model RNA containing hairpin 92.²¹,²⁵ Although the reference cell’s linear RNA is not predicted to form a secondary structure, to ensure that both RNA molecules were treated the same way, this RNA molecule was also taken through the annealing process described above.

The unconjugated streptavidin was removed from the chips’ surface by three injections of a solution containing 1 M NaCl and 50 mM NaOH at 30 μL min⁻¹, as suggested by the GE Healthcare Life Sciences. Before injection into the flow cells, RNA samples were diluted 200-fold in SPR running buffer, which consisted of 50 mM HEPES–KOH pH 7.5, 5 mM MgCl₂, 5% glycerol, 0.5% Tween-20, 1 mM dithiothreitol (DTT), and various concentrations of KCl. The DbpA protein’s storage buffer was exchanged with the SPR running buffer using Microcon MWCO 10 kDa centrifugal filters (Millipore Corporation, Billerica, MA). The extinction coefficient of 26 970 M⁻¹ cm⁻¹ at 280 nm was used to determine the DbpA protein’s concentration.

The equilibrium and kinetics of DbpA–RNA complex formation were investigated using a Biacore X100. The RNA was immobilized to 50 or 250 response units (RU), which corresponds to an RNA concentration of 34.5–172.4 μM.³⁵ However, the volume of the matrix is only about 120 pL, and a concentration of 34.5–172.4 μM correspond to 4–20 femtomoles of molecules in the matrix. The flow rate used for RNA immobilization and the protein–RNA binding experiments was 30 μL min⁻¹. All of the experiments were carried out at 22 °C. The measurements were performed by the addition of several concentrations of DbpA for 180 s, and the complex dissociation was monitored for 360 s.

The resulting sensograms for the reference and the experimental flow cell were zeroed on the x axis to mark the beginning of injection with respect to each other. To correctly determine the differences in response between the reference and the experimental cell, prior to DbpA injection, sensograms were also zeroed on the y axis.²⁷,³⁶

To determine the DbpA association and dissociation rate constants to RNA, the equations below (eqs 1–4) were employed to simultaneously fit the response. BIAevaluation software (version 4.1) was used for these fits.

\[
\frac{d[RNA]}{dt} = -(k_f[RNA][DbpA] - k_d[RNA-DbpA]) \tag{1}
\]

\[
\frac{d[RNA-DbpA]}{dt} = (k_f[RNA][DbpA] - k_d[RNA-DbpA]) \tag{2}
\]

In eq 5, \( R_{eq} \) is the sensogram response at the equilibrium, the \( R_{max} \) is the maximum response, and \( K_D \) is the dissociation constant. BIAevaluation software (version 4.1) was used to fit the sensogram data and calculate \( K_D \).

**ATPase Assay.** The dependence of the DbpA ATP hydrolysis on ATP concentration (Figure 1A) was measured by pyruvate kinase/lactate dehydrogenase-coupled assay as previously described.²⁹ In this assay, ATP hydrolysis is coupled with the oxidation of NADH to NAD⁺. The formation of NAD⁺ produces a decrease in absorbance reading at 338 nm. The measurements were performed at 22 °C and using 96-well plates. The ATPase reactions were performed in the presence of 2 μM RNA, 30 nM DbpA, 50 mM HEPES (pH 7.5), 50 mM KCl, 5 mM MgCl₂, 0.5% (v/v) Tween-20, 1 mM dithiothreitol (DTT), 1 mM phosphoenolpyruvate, 0.250 mM NADH, 9 units mL⁻¹ pyruvate kinase, 13 units mL⁻¹ lactate dehydrogenase, and 0–5 mM ATP-Mg concentrations. The RNA molecule used for these experiments had the same sequence as the RNA shown in Figure 1A, but was nonbiotinylated. RNA was annealed through the same temperature cycles and in the same buffer condition as RNA used for the SPR experiment. Similar to the SPR experiments, these experiments were also performed at 22 °C. The Michaelis–Menten equation was used to fit the data. \( k_{in} \) (Michaelis–Menten constant) and \( k_{cat} \) (the turnover number) were obtained from the fit.

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**Notes**

The authors declare no competing financial interest.

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