Corrections

**PHYSIOLOGY**

The authors note that the author name Jared D. Gorham should have appeared as James D. Gorham. Additionally, the author name Victor G. Bundoc should have appeared as Virgilio G. Bundoc. The corrected author line appears below. The online version has been corrected.

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**GENETICS**

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**BIOCHEMISTRY**

The authors note that due to a printer’s error, several of the Supporting Figures were referenced incorrectly in the main text. All references to Supporting Figure 3 should have instead referred to Supporting Figure 5, and all references to Supporting Figure 5 should have instead referred to Supporting Figure 3. All references to Supporting Figure 4 should have instead referred to Supporting Figure 6, and all references to Supporting Figure 6 should have instead referred to Supporting Figure 4. The online version has been corrected.

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Pathway of ATP utilization and duplex rRNA unwinding by the DEAD-box helicase, DbpA

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DEAD-box RNA helicase proteins use the energy of ATP hydrolysis to drive the unwinding of duplex RNA. However, the mechanism that couples ATP utilization to duplex RNA unwinding is unknown. We measured ATP utilization and duplex RNA unwinding by DbpA, a non-processive bacterial DEAD-box RNA helicase specifically activated by the peptidyl transferase center (PTC) of 23S rRNA. Consumption of a single ATP molecule is sufficient to unwind and displace an 8 base pair rRNA strand annealed to a 32 base pair PTC-RNA “mother strand” fragment. Strand displacement occurs after ATP binding and hydrolysis but before P_i product release. P_i release weakens binding to rRNA, thereby facilitating the release of the unwound rRNA mother strand and the recycling of DbpA for additional rounds of unwinding. This work explains how ATPase activity of DEAD-box helicases is linked to RNA unwinding.

Results and Discussion

We investigated how DbpA couples ATPase activity to PTC-rRNA duplex (dsRNA) unwinding by correlating individual ATPase cycle transitions with 8-mer rRNA strand replacement. The rRNA substrate (Fig. S1A) consists of a fluorescently-labeled 8-mer rRNA strand annealed to a 32-mer mother strand comprised of hairpin 92 and helix 91 of domain V of the PTC (5). Duplex strand displacement was measured in real time by following the changes in fluorescence anisotropy and intensity of the fluorescently-labeled 8-mer RNA strand that occur after mixing DbpA-dsRNA complex with excess adenine nucleotide (ATP, AMPPNP, ATPγS, or ADP; Fig. L4). Unwound 8-mer strand has a lower fluorescence anisotropy and higher fluorescence intensity than 8-mer annealed to 32-mer PTC-RNA (Fig. L4 and B). Rapid duplex strand displacement is ATP-dependent (Fig. L4) and, therefore, should be linked to DbpA ATPase activity. Strand displacement also occurs with fluorescently-labeled mantATP (Fig. S2). Little unwinding occurs within 10 s in the absence of nucleotide or when ADP or the non- or slowly-hydrolysable ATP analogs, AMPPNP and ATPγS are present (Fig. L4), consistent with previous measurements made using a different assay system (5). However, ATP-independent unwinding of a small fraction of the total duplex RNA occurs slowly on the minute timescale, and it results from the preferential binding of the protein to unwind PTC-rRNA (Fig. L4 and Table S1).

ATP-dependent 8-mer strand displacement is completed within 2 s (Fig. L4). The observed unwinding rate constants depend on the [DbpA] (at saturating [Mg-ATP]; Fig. 1C) and [Mg-ATP] (when [DbpA] is near saturating) (Fig. 1D) and reach a maximum (k_unwind) of ~3 s⁻¹. A fraction of the 8-mer RNA duplex remains as duplex, because the observed final anisotropy value is slightly higher than that of free 8-mer RNA (Fig. L4). The fraction of duplex RNA during steady-state ATP cycling (20%) depends on the RNA concentration and results from reannealing of the 8-mer fragment (Table S1) as well as a small (<5%) fraction of dsRNA that is non productive for unwinding by DbpA, presumably due to alternative RNA folding and annealing, as previously reported (5). Negligible changes in fluorescence anisotropy or fluorescence intensity occur (change in anisotropy units of ≤0.01) on ATP-induced unwinding of DbpA-dsRNA complex when the 32-mer mother strand is fluorescently labeled instead of the 8-mer (Fig. S1B), indicating that DbpA remains bound to the 32-mer product after unwinding under our conditions, as predicted from the binding affinity (K_D = 300 nM) (Table S1, Fig. S5, and ref. 9).

DbpA binds PTC-RNA and cycles (i.e., hydrolyzes ATP) as a monomer (10, 11). The observations that the ATPase cycle rate constants (12), including the reversibility of ATP hydrolysis as assayed by oxygen isotope exchange (Fig. S6), are independent of the [DbpA] are consistent with this interpretation. These results together with the observation that the ATPase cycling and unwinding rate constants are comparable at ~3 s⁻¹ favor a mechanism in which monomeric DbpA displaces the 8-mer fragment during a single catalytic turnover of ATP (i.e., DbpA consumes a single ATP molecule per 8-bp unwinding event as suggested for unwinding of a 6–7 bp with similar duplex stability by Cytt9) (13). The energy needed to unwind the 8-mer fragment (~40.5 kJ mol⁻¹, determined by using the program RNA Structure) (14) is comparable to that available from hydrolysis of a single ATP under our conditions (ΔG° ~–40 kJ mol⁻¹). However, a mechanism in which DbpA unwinds <8 base pairs of the fragment and spontaneous dissociation of the remaining unstable duplex region follows is likely (13, 15).
To determine when during the ATPase cycle (e.g., ATP binding, hydrolysis, and/or product release) duplex strand displacement occurs, we measured the individual rate and equilibrium constants of the RNA-activated DbpA ATPase cycle (Scheme 1, Table S1, and ref. 12) and correlated time courses of changes in chemical state with unwinding transitions. These measurements of steady-state ATPase, nucleotide binding, ATP hydrolysis, and product release were performed with two different RNAs, one corresponding to the dsRNA substrate and the other to ssRNA mother strand unwinding product (Fig. S1). The regions flanking the hairpin 92 component (ssRNA or dsRNA of helix 91) (Fig. S1C and D) are required for DbpA ATPase activity (16).

Nucleotide binding and dissociation were measured using fluorescence-labeled mant-nucleotides (mantATP and mantADP) (12, 17). ATP hydrolysis and $\text{P}_i$ release were determined by using $^{18}\text{O}$ isotopic exchange, chemical quench flow, and the fluorescently-labeled phosphate binding protein (12, 17).

ATP and ADP binding, ATP hydrolysis, and $\text{P}_i$ release are comparable when bound to dsRNA substrate or ssRNA product (Table S1) as well as a longer 153-mer PTC-RNA substrate (12). The observation that ATPase activity also occurs with unwound product indicates that ATP utilization is independent of duplex unwinding and that parallel ATPase pathways along single- and double-stranded RNA exist (Scheme 1). ATPase activity on single-stranded RNA may result from ATP-dependent translocation that is independent of unwinding as occurs with some DNA helicases (18), although it has been argued that this does not occur with DEAD-box proteins (19). Such activity could be used by DbpA for another function such as searching for distal dsRNA regions on rRNA required for unwinding and/or remodeling. However, a DbpA ATPase activity linked to RNA translocation would not affect any conclusions or interpretations because 8-mer strand displacement occurs during the first ATP turnover.

Fig. 1. Kinetics of DbpA catalyzed 8-mer strand displacement. A. Time courses of 8-mer strand displacement assayed from changes in fluorescence anisotropy after mixing an equilibrated mixture of 4,000 nM DbpA and 200 nM dsRNA with buffer, 2 mM ADP, AMPPNP or ATP$\gamma$S (Red Curve), or 2 mM MgATP (Blue Curve). The time courses with ADP, AMPPNP, or ATP$\gamma$S were experimentally indistinguishable given the slow reaction and spectroscopic signal. B. Time courses of unwinding assayed from changes in total fluorescence intensity after mixing an equilibrated mixture of 200 nM dsRNA and DbpA (Curves A–H represent 27, 54, 108, 162, 432, 1296, 4030, and 4330 nM DbpA, respectively) with 5 mM MgATP. C. [DbpA]-dependence of the duplex RNA unwinding rate constant ($k_{\text{unwind}}$) in the presence of 5 mM ATP; the [dsRNA] is 200 nM. D. [ATP]-dependence of $k_{\text{unwind}}$; the [dsRNA] is 200 nM and the [DbpA] is 4100 nM. The Lines in C. and D. are the best fits of the data to rectangular hyperbola.

Knowledge of the ATPase cycle transition rates and equilibrium constants (Table S1) permits the determination of the time-dependent formation of populated biochemical intermediates and reaction flux through the cycle (Fig. 2). These data are presented in the supporting online material, but they are essential to the arguments that follow. ATP binding is rapid and completed within milliseconds (Fig. 2, Fig. S3), followed by the formation of an ATP hydrolysis competent state (12) that precedes and limits rapid and reversible ATP hydrolysis, and then Pi and ADP product release (Fig. 2, Fig. S4). The rates of the biochemical transitions limiting ATP hydrolysis and Pi release are comparable, so Pi release time courses display a prominent lag phase (Fig. 2, Fig. S4, and ref. 12). ADP dissociates rapidly (Fig. S3 and Fig. S4) after Pi release and the cycle begins again with rapid ATP binding to the nucleotide-free DbpA-RNA complex. The overall RNA affinities depend on the DbpA bound nucleotide; “strong” binding occurs with bound ADP-Pi (Table S1).

The pathway of ATP-dependent dsRNA unwinding by DbpA is revealed by comparing the time courses of 8-mer strand displacement with those of the individual ATPase cycle transitions (Fig. 2). Duplex strand displacement occurs after ATP binding and hydrolysis, but before Pi and ADP release (Fig. 2). This conclusion is supported by the observation that strand displacement with mantATP occurs ~2-fold more slowly than with ATP at an identical concentration (Fig. S2) because the conformational change that precedes and limits rapid ATP hydrolysis is ~2-fold more slowly with mantATP than unlabeled ATP (12). We can eliminate pathways in which 8-mer strand displacement by DbpA coincides with Pi release because strand displacement is more rapid than Pi dissociation and also occurs without a detectable lag phase. Similarly, we can eliminate a pathway in which unwinding occurs with ATP binding, as suggested for the DEAD-box proteins Ded1 (20), because it is much more rapid than unwinding. The large free energy change associated with ATP binding to DbpA must be used to induce a conformational change of the helicase-RNA complex (12, 27).

A mechanism in which 8-mer strand dissociation occurs rapidly (~100 s⁻¹) from the ADP-Pi conformation of DbpA (Scheme 1 Red Box and ref. 12), accounts for the experimental data (Fig. S7), including the ATPase cycle rate and equilibrium constants and observed unwinding rate constants (Scheme 1). Duplex unwinding competes with ATPase cycling along the dsRNA pathway. ATP hydrolysis promotes the formation of a DbpA conformation(s) that facilitates strand dissociation, a diffusive process driven by Brownian motion. We favor a mechanism in which DbpA with bound ADP-Pi binds the unwound helix of PTC-RNA strongly and distorts the double strand RNA hydrogen bonding (15) to such an extent that the annealed 8-mer fragment dissociates (Fig. 3). That is, DbpA behaves as a nucleotide-dependent helix destabilizing protein. Release of Pi weakens the DbpA affinity for RNA, thereby facilitating release of the
unwound tRNA mother strand and recycling DbpA for another round of unwinding. Such a strong to weak RNA binding transition would precede detachment during stepping of processive, translocating helicases. Reannealing of longer, partially unwound RNA duplexes will contribute to futile ATPase cycling without strand displacement (12).

Conclusion
Rapid and efficient tRNA unwinding by the DEAD-box protein, DbpA, requires ATP binding and hydrolysis. Strand displacement occurs after hydrolysis of bound ATP and formation of the “strong” RNA binding conformation of DbpA. The kinetic stability of the ADP-Pi state arising from a slow and partially rate-limiting Pi release (Table S1 and ref. 12) ensures that strand dissociation occurs before Pi is released. Pi release weakens the RNA binding affinity, facilitates dissociation from the unwound rRNA mother strand and recycles DbpA for additional rounds of unwinding.

It should be noted that a kinetic competition between strand dissociation and progression through the ATPase cycle exists at every nucleotide state (Scheme 1). Dissociation of the 8-mer strand is far more rapid in the ADP-Pi unwinding. Such a strong to weak RNA binding transition would precede detachment during stepping of processive, translocating helicases. Reannealing of longer, partially unwound RNA duplexes will contribute to futile ATPase cycling without strand displacement (12).

Materials and Methods

Reagents and Purification of DbpA. All chemicals and reagents were the highest purity commercially available. Millipore MiliQ® dispensed water that had been treated with DEPC for 8 h and autoclaved was used in all procedures. RNase activity was undetectable in all reagents, buffers, and protein preparations. ATP and ATP-γS were purchased from Roche Molecular Biochemicals. Adenosine 5′-(γ-3H)imidodiphosphate (AMPγP) and ADP were purchased from Sigma. A molar equivalent of MgCl2 was added to nucleotides immediately before use.

Recombinant DbpA was overexpressed, purified, and concentration determined as described (4). All measurements were made at 25 °C in (final conditions) Km75S buffer (20 mM K+ - HEPES (pH 7.5), 75 mM KCl, 5 mM MgCl2, and 1 mM DTT). The concentration of ATP with bound Mg2+ (Mg-ATP) was calculated from the Mg-ATP binding affinity (23) and the total nucleotide and cation concentrations present in solution (22).

RNA Substrates Used in This Work. RNAs used in this work were commercially purchased from Dharmacon and deprotected as instructed. The RNA unwinding substrate (Fig. S1) was prepared by hybridizing 20 μM of the 8-mer and 32-mer ssRNA species (8-mer: 5′-GACGCUUCUGGCUCU-3′; 32-mer: 5′-CCUUCAGGUAGGGGGUAAAGGGGCUUCUGGC-3′) in hybridization buffer (4, 5).

Pre-Steady-State Kinetic Measurements. All transient kinetic fluorescence measurements were made in Km75S buffer using an Applied Photophysics SX20 stopped-flow apparatus thermostatted at 25 ± 0.1°C. Excitation of fluorescein labeled RNA substrates was done at 485 nm using a Xenon lamp light source and the emission monitored at 590° through a 515 nm long-pass colored glass filter. Concentrations stated are final after mixing.

Fluorescence anisotropy measurements were performed with T-format excitation and emission arrangement. Samples were excited at λex = 485 nm by using vertical polarized light. The emitted vertical and horizontal polarized light was monitored at 90° through a 515 nm long-pass colored glass filter. G-factor for correction of the different gain between the dual Photomultiplier Tube detectors was calculated as described by the instrument manufacturer. The fluorescence intensity (S) and total anisotropy (A) are given by:

\[
S = \frac{(G_1 - 2I_i)}{(G_2 + 2I_i)} \tag{[1]}
\]

\[
A = \frac{(G_1 - I_i)}{(G_2 + I_i)} \tag{[2]}
\]

Whereas in a kinetic experiments the fluorescence intensity S(t) and total anisotropy A(t) are given by:

\[
S(t) = \sum_{i} \chi_i(t) q_i \tag{[3]}
\]

\[
A(t) = \sum_{i} \chi_i(t) q_i \tag{[4]}
\]

Where \(\chi_i\), the fraction of total observed species in state \(i\) at time \(t\), \(q_i\) is the effective quantum yield of state \(i\), and \(r_i\) represents the steady state anisotropy values of species \(i\). The total fluorescence and anisotropy were fitted globally to a one step reaction mechanism as detailed by Otto et al. (23).

Real Time Duplex RNA Unwinding Assay. Unwinding reactions were carried out by rapidly mixing an equilibrated mixture of DbpA (at the indicated concentration) and 200 nM bimolecular dsRNA unwinding substrate with ATP (at the indicated concentration). Bimolecular RNA was at 200 nM and the [DbpA] and [ATP] as varied as indicated below. Control experiments confirm that ~95% of the duplex RNA bound to DbpA remains as duplex prior to mixing with ATP. Mixing the reactants in different sequence had no effect on the overall observed unwinding rate. Labeling the 8 mer ssRNA (displacement strand) either in the 5′ or 3′ does not change the resolved unwinding intermediates and the observed unwinding rate. The reannealing rates of the 8 mer ssRNA to the mother strand (32 mer), DbpA-mother strand, and DbpA-mother strand-ADP complexes were determined independently. The kinetic rate constants for reannealing were incorporated to the kinetic models to account for reannealing of unwound 8-mer strand during unwinding.

Kinetic Simulations and Modeling. Simulations of reaction time courses and equilibrium distribution of biochemical states were performed with Tenua (provided by D. Wachstock, available free at http://www.geocities.com/tenua4java/) and/or Kintek Explorer (24, 25) both of which are based on the kinetic simulation program KINSIM developed by Carl Frieden and colleagues (26). Table S1 shows the kinetic parameters used for the construction of Fig. 2 and in the simulation of the unwinding reaction kinetics as a function of [DbpA] to fit the experimental data. The nomenclature of the rate constants are according to Scheme 1.

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