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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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 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 have investigated how DbpA couples ATPase activity to PTC-RNA 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 rRNA strand that occur after mixing DbpA-dsRNA complex with excess adenosine nucleotide (ATP, AMPPNP, ATP$_\gamma$-S, 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 ATP$_\gamma$-S 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$_{\text{unwind}}$) of $\sim$3 s$^{-1}$. A fraction of the 8-mer rRNA 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 $\leq$0.01) on ATP-induced unwinding of DbpA-dsRNA complex when the 32-mer mother strand is fluorescently labeled instead of the 8-mer (Fig. S1B), indicating that DbpA remains bound to the 32-mer product after unwinding under our conditions, as predicted from the binding affinity ($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 $\sim$3 s$^{-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 8-8 bp unwinding event as suggested for unwinding of a 6–7 bp with similar duplex stability by Cytl9) (13). The energy needed to unwind the 8-mer fragment ($\sim$40.5 kJ mol$^{-1}$, determined by using the program RNA Structure) (14) is comparable to that available from hydrolysis of a single ATP under our conditions ($\Delta$G$^{\text{unw}}$ $\sim$40 kJ mol$^{-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).


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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. S1 C and D) are required for DbpA ATPase activity (16). Nucleotide binding and dissociation were measured using fluoroscently-labeled mant-nucleotides (mantATP and mantADP) (12, 17). ATP hydrolysis and P<sub>i</sub> release were determined by using 18<sup>O</sup> isotopic exchange, chemical quench flow, and the fluoroscently-labeled phosphate binding protein (12, 17).

ATP and ADP binding, ATP hydrolysis, and P<sub>i</sub> 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 ATPγS (Red Curve), or 2 mM MgATP (Blue Curve). The time courses with ADP, AMPPNP, or ATPγS 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<sub>unwind</sub>) in the presence of 5 mM ATP; the [dsRNA] is 200 nM. D [ATP]-dependence of k<sub>unwind</sub>; 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.](image)

![Scheme 1. Matrix of all possible paths for ATPase linked duplex RNA unwinding by DbpA. The pathway of 8 bp duplex RNA unwinding is indicated by the Red Box. H: helicase, dr: double-stranded RNA substrate, sR: “mother strand” ssRNA product, sP: displaced 8-mer strand, T: ATP, D: ADP, P<sub>i</sub>: inorganic phosphate.](image)
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 P$_i$ and ADP product release (Fig. 2, Fig. S4). The rates of the biochemical transitions limiting ATP hydrolysis and P$_i$ release are comparable, so P$_i$ release time courses display a prominent lag phase (Fig. 2, Fig. S4, and ref. 12). ADP dissociates rapidly (Fig. S3 and Fig. S4) after P$_i$ 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-P$_i$ (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 P$_i$ and ADP release (Fig. 2). This conclusion is supported by the observation that strand displacement with manATP 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 manATP than unlabeled ATP (12). We can eliminate pathways in which 8-mer strand displacement by DbpA coincides with P$_i$ release because strand displacement is more rapid than P$_i$ 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-P$_i$ 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-P$_i$ 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 P$_i$ weakens the DbpA affinity for RNA, thereby facilitating release of the

Fig. 2. Relationship between ATP utilization and duplex RNA unwinding by DbpA. Cumulative ATP binding to DbpA-dsRNA complex (Black), ATP hydrolysis (Red) and P$_i$, and ADP product release (Green) reactions as defined by Scheme 1 computed from the experimentally determined rate and equilibrium constants (Table S1 and S2) under reaction conditions of 4.3 μM DbpA, 5 mM ATP, and 200 nM dsRNA. The fit of the 8-mer strand displacement (Blue) time course obtained under identical conditions (Fig. 1) is shown for comparison.

Fig. 3. Pathway of ATP-dependent duplex rRNA unwinding by DbpA. The depicted scheme corresponds to a single turnover unwinding event (Fig. 1 and Scheme 1) starting from a preformed DbpA-dsRNA complex. The DbpA core is Blue and the C-terminal domain is Purple. Weak to strong RNA binding states are box colored Light Green to Dark Green, resp. For simplicity, the RNA detached states and an activated, ATP hydrolysis competent, strong RNA binding DbpA-ATP-RNA complex formed after isomerization of the DbpA-RNA-ATP complex (12) are not shown.
unwound rRNA 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 rRNA 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-P state arising from a slow and partially rate-limiting P release (Table S1 and ref. 12) ensures that strand dissociation occurs before P is released. P 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-P state than in all other or no nucleotides bound states of DbpA (Tables S1 and S2). If progression through the ATPase cycle is inhibited, as can be achieved with non-hydrolyzable ATP analogs, strand dissociation through an otherwise “off-pathway” reaction is favored. In the presence of ATP, these branched off-pathway, strand displacement reactions are not followed because progression through the cycle is far more rapid (Tables S1 and S2). However, when progression through the cycle is inhibited (i.e., with analogs), these off-pathway reactions dominate because they now have a greater probability (i.e., faster rate constant) than progression through the ATPase cycle. This behavior could explain why unwinding by some DEAD box RNA helicases is observed with non-hydrolyzable ATP analogs (20), but considerably more slowly than the ATP-dependent reaction. Similarly, progression through an off pathway reaction could be favored under low [MgCl2] conditions (13) because ATP hydrolysis will presumably occur less readily. However, strand displacement is only several-fold slower in the presence of low magnesium (13), suggesting the existence of an ATP-bound intermediate that is capable of partially accelerating unwinding, as suggested (12, 13).

Materials and Methods
Reagents and Purification of DbpA. All chemicals and reagents were the highest purity commercially available. Millipore MilliQ® 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-S were purchased from Roche Molecular Biochemicals. Adenosine 5’-(γ-32P)triphosphate (AMPpNP) and ADP were purchased from Sigma. A molar equivalent of MgCl2 was added to nucleotides immediately before use.

Recombinant DbpA was overexpressed, purified, and concentrated as described (4). All measurements were made at 25°C in (final conditions) KMg75 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 (21) 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 Dharmaco 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′-F*-CUCUUUGC where F* represents the effective quantum yield of state i, and qi 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. Wachsstock, 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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