Ded1p, a DEAD-box Protein Required for Translation Initiation in Saccharomyces cerevisiae, Is an RNA Helicase*

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The Ded1 protein (Ded1p), a member of the DEAD-box family, has recently been shown to be essential for translation initiation in Saccharomyces cerevisiae. Here, we show that Ded1p purified from Escherichia coli has an ATPase activity, which is stimulated by various RNA substrates. Using a RNA strand-displacement assay, we show that Ded1p has also an ATP-dependent RNA unwinding activity. Hydrolysis of ATP is required for this activity: the replacement of ATP by a nonhydrolyzable analog or a mutation in the DEAD motif abolishing ATPase activity results in loss of RNA unwinding. We find that cells harboring a Ded1 protein with this mutated DEAD motif are nonviable, suggesting that the ATPase and RNA helicase activities of this protein are essential to the cell. Finally, RNA binding measurements indicate that the presence of ATP, but not ADP, increases the affinity of Ded1p for duplex versus single-stranded RNA; we discuss how this differential effect might drive the unwinding reaction.

DEAD-box proteins form a large family of putative RNA helicases that show sequence similarity to eIF4A, a eukaryotic translation initiation factor. All members of the DEAD-box family share an eight conserved amino acid motifs, including the characteristic sequence Asp-Glu-Ala-Asp (DEAD in the single-letter code) that inspired their name (1). Sequence comparisons identified the closely related DEAH- and DExH-box families, which together with the DEAD-box proteins, form the helicase superfamily II (2). The DEAH and DExH families notably include DNA helicases involved in DNA replication and recombination. The putative RNA helicases of superfamily II are found in a wide range of organisms, including bacteria, viruses, and eukaryotes ranging from yeast to humans. Although they are involved in very diverse cellular functions, such as pre-mRNA splicing, rRNA processing, and mRNA export, translation, and decay, they are all supposed to share in common an RNA helicase activity (3, 4). This activity has been inferred from the ability of eIF4A to melt out mRNA structure (5) or to dissociate an RNA duplex in vitro (6), in an ATP-dependent manner. However, although NTPase activity has been demonstrated for all purified DEAD-box and related proteins, RNA helicase activity has been characterized for only a few of them and remains conjectural in most cases.

Nevertheless, numerous steps of gene expression are likely to require RNA helicase activity, either to unwind RNA secondary structures or to rearrange large RNA structures, or even to disrupt RNA-protein interactions. For example, transient base pairings between small nuclear RNAs and between small nuclear RNAs and pre-mRNA, which occur during pre-mRNA splicing, are often mutually exclusive and thus need to form and dissociate sequentially. At least eight DEAD-box and related proteins have thus far been shown to be required for splicing in yeast and may accomplish these structural rearrangements (7). Similarly, 13 DEAD-box are assumed to be involved in extensive rearrangements between pre-rRNA and ribosomal proteins/small nucleolar RNAs during ribosome biogenesis (8).

Translation initiation in eukaryotes also presumably requires removal of secondary structure in the 5'-untranslated region of mRNAs for the binding of the small ribosomal subunit and its migration toward the AUG codon ("scanning") (9). Indeed, insertion of stable stem-loop structures in the 5'-untranslated region of mRNAs inhibits translation initiation in both higher eukaryotes (e.g. 10, 11) and yeast (e.g. 12, 13). In mammals, unwinding of these structures has been attributed to the cap-binding complex eIF4F, because it shows an RNA helicase activity in vitro (6), and its overexpression in vivo facilitates translation of mRNAs with highly structured 5'-untranslated regions (14). This complex consists of eIF4E, which binds to the cap structure at the 5'-end of mRNAs, eIF4G, and eIF4A, which is the active helicase component. Another factor, eIF4B, may also be involved in the unwinding process because it is required for the helicase activity of eIF4A in vitro (6, 15).

Despite the high degree of conservation of translation in eukaryotes, the cap-binding complex in the yeast Saccharomyces cerevisiae is not equivalent to mammalian eIF4F, because yeast eIF4A is not found in the complex (16). Moreover, yeast eIF4A has been shown to be active in RNA unwinding in vitro with mammalian eIF4B (17), but not with its putative yeast counterpart, Tif3p (18), suggesting that additional yeast factors are required to catalyze this reaction. We and others (19, 20) have recently described another DEAD-box protein, Ded1p, required for translation initiation in S. cerevisiae. We isolated the DED1 gene as a multicopy suppressor of a temperature-sensitive mutation in eIF4E. Analyses of its suppressor activity, of polysome profiles of ded1 mutant strains, and of synthetic lethal interactions with different translation initiation mutants indicated that Ded1p has a role in translation initiation (19). Consistently, immunodepletion of Ded1p in an in vitro translation system abolished translation activity (20). Genetic data suggest that Ded1p and eIF4A play independent roles in translation initiation, but the nature of these roles is yet unknown. Here, we investigate the biochemical activities of...
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Ded1p and show that it has RNA-dependent ATPase and ATP-dependent RNA helicase activities, both activities depending on the integrity of the DEAD motif. RNA binding studies also show that the affinity of Ded1p for RNA can be modulated by virtue of a change in ATP hydrolysis.

EXPERIMENTAL PROCEDURES

Strains—The S. cerevisiae strains used in this study are derivatives of W303 (MATa/α, ura3-1, ade2-1, his-11, 15his-11B, trp1-3, 112his-3, 112 trpl-1/trpl-1). The strain ID2 (MATa/α, MATa DED1/DED1::HIS3MX6) is a meiotic segregant of ID2 that requires a plasmid-borne copy of DED1 for viability. The inability of ID2-2A to lose the resident YCplac111-Ded1p plasmid (19) was tested by plasmid shuffling (22), using 5-fluoro-orotic acid plates. Standard yeast genetic techniques and media were as described (23).

Expression and Purification of Ded1p—To express an N-terminal His6-tagged Ded1 fusion protein from its cognate promoter, a fusion polymerase chain reaction was performed (24). Briefly, two fragments with sequence overlap were generated in a first polymerase reaction series with YCplac33-DED1 as a template and the oligonucleotides 5’-GCATGCTGCACTCCGTG-3’ (where ë refers to outer oligonucleotide) and 5’-GTGATGGTGATGGTGCATAGATGAGAATGCTGGTTG-3’ (the DED1 homology region, external oligonucleotide) with EcoRI and BamHI restriction sites. After conversion of the mix (0.5M KCl, 50 mM Tris-HCl, pH 7.9, 0.5 mM dithiothreitol, 10% glycerol), the resulting plasmid (labeled DED1::His6-Ded1p) in a lysis buffer with 1M NaCl, 50 mM Tris-Cl, 0.5 mM dithiothreitol, sonicated, and centrifuged (30 min at 4°C). The supernatant was loaded on a 1 ml nickel-nitrilotriacetic acid column (Qiagen). After washing with 30 and 60 mM imidazole, the His6-Ded1p eluted with 250 mM imidazole. The eluted fractions were adjusted to 50% glycerol and stored at −20°C. We usually obtained around 0.6 mg of Ded1p from 200 ml of culture. The protein has a tendency to precipitate; where necessary, precipitated protein was removed by centrifugation.

Construction of the DAAD Mutant—To change the glutamic acid residue of the DEAD motif into alanine, the pET22b-His6-Ded1 plasmid was mutated in vitro with the QuickChange site-directed mutagenesis kit of Stratagene. A 613 nt-long BamHI-BstFI fragment from candidates carrying the desired mutation was entirely sequenced and cloned into the BamHI-BstFI-restricted original pET22b-His6-Ded1 plasmid to eliminate eventual mutations outside the fragment. The mutant protein was purified as for the wild-type. The Ndcl-SalI fragment from the mutated pET22b-His6-Ded1 plasmid was cloned into the Ndcl-SalII-restricted YCplac111-His6-Ded1 plasmid.

ATPase Assay—ATPase activity was monitored continuously by a coupled spectrophotometric method (26). This method uses pyruvate kinase and lactate dehydrogenase to link hydrolysis of ATP to oxidation of NADH, which results in a decrease in the absorbance at 345 nm. Assays were performed at 37°C in a reaction volume of 0.4 ml, in buffer containing 20 mM Tris-HCl, pH 8, 50 mM KCl, 5 mM MgCl2, 1 mM dithiothreitol, 1 mM ATP, 300 µM NADH, 2 mM phosphoenolpyruvate, and 3 units/ml of pyruvate kinase and lactate dehydrogenase. RNA and protein were added as indicated in Figs. 2 and 3. Absorbance data were analyzed using Kaleidagraph 3.0 (Synergy). The steady-state rate of ATP hydrolysis equals that of NADH oxidation, which was quantified using 6300 m-1 cm-1 for the extinction coefficient of NADH.

RNA Preparations—Total RNA from W303 yeast strain was extracted by the acid-phenol method (27). Poly(A)+ (polyadenylated) RNA was prepared from total RNA using an oligo(dT) column. The 18 and 25 S rRNAs were prepared from 40 and 60 S ribosomal subunits that were separated by centrifugation on a sucrose gradient as described in Ref. 19. Their purity was checked by gel electrophoresis. Poly(A), poly(C), poly(G), and poly(U) homopolymers and yeast tRNAs were purchased from Sigma.

RNA substrates used for helicase and RNA binding assays were prepared as described (28). Briefly, two transcripts of 43 and 68 nucleotides were synthesized in vitro from pGEM-3Z and pGEM M012 vectors, the shorter one being labeled with [α-32P]GTP (specific activity, 2.5 × 109 cpm/µmol). The nucleotide sequences of the two strands are as follows: 43-nucleotide strands: 5′-AAAGACCGAGGCAAGGACUGAAGGACAGGAGGCGGCUCCUCUGAGU-3′ (duplex region is underlined). These transcriptions were annealed and the resulting partial duplex, which consists of a 14-base pair region flanked by 5′ ss overhangs of 29 and 54 nucleotides (see Fig. 4A), was purified by native polyacrylamide gel electrophoresis. Its specific activity was calculated to be 2.5 × 109 cpm/µmol. We also constructed a 55-base pair “complete” duplex by transcribing a 64-nucleotide RNA synthesized by SP6 RNA polymerase from EcoRI-restricted pGEM3 plasmid (labeled with [α-32P]GTP) and a 60-nt RNA synthesized by T7 RNA polymerase from Hind III-restricted pGEM3 plasmid.

RNA Helicase Assay—The activity was assayed in the presence of 20 mM Tris-HCl, pH 8.0, 70 mM KCl, 2 mM MgCl2, 2 mM dithiothreitol, 15 units RNaseE activity (25). Transformed cells were grown and induced with 0.5 mM IPTG. RNA substrates for the binding reactions were added at 10 µg/ml for RNA helicase activity assays. The ATPase reactions were initiated by addition of ATP, as indicated in Fig. 4. After incubation for 10 min at 37°C, the reactions were stopped by the addition of 4 µl of a solution containing 1.2% SDS, 10 mM EDTA, 40% glycerol, bromphenol blue, xylene cyanol, and 250 µg/ml proteinase K. For positive controls (cf. Fig. 4, lane 1), duplex RNA was separated by electrophoresis at 95°C for 2 min, followed by rapid cooling in ice. Samples were electrophoresed on a 10% polyacrylamide gel in 0.5x TBE. Labeled RNAs were visualized by autoradiography and quantified using a Fuji BAS 1000 phosphorimager. The percentage of unwinding was calculated using the formula (1−(monomer/total)) (monomer/total) × 100, where “total” is the amount of monomer plus dimer, and ø refers to the numbers observed in absence of ATP.

RNA Filter Binding Assay—RNA substrates for the binding reactions were either the duplex used in the helicase assay, or the labeled 43-nt transcript used to construct this duplex, or, in some experiments, the complete duplex. Reaction mixtures (20 µl) contained 20 mM Tris-HCl, pH 8.0, 70 mM KCl, 2 mM MgCl2, 2 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, 40 fmol (2 nm) of RNA, and varying concentrations of Ded1p. When present, nucleotides (ADP and AMP-PNP (Sigma)) were used at a final concentration of 2 mM. Samples were incubated for 5 min at room temperature before filtering. A “double-filter” assay was performed following the method of Ref. 29, using a dot-blot apparatus. The RNA-protein complexes were retained on nitrocellulose (Schleicher & Schuell), whereas free RNA was retained on a charged nylon membrane (Hybond-N+, Amersham Pharmacia Biotech) placed beneath. Membranes were washed before and after loading the samples in 1 ml of ice-cold reaction buffer. The amount of RNA present on each membrane was quantified using a Fuji BAS 1000 phosphorimager, and the fraction of RNA bound to the protein was determined. All assays were corrected for the small fraction of bound RNA in absence of protein (on average, 1.5%). Binding curves were obtained by plotting the fraction of bound RNA versus protein concentration.

RESULTS

Purification of Ded1p—To facilitate the biochemical characterization of Ded1p, we overexpressed it in E. coli as a fusion protein with a 6-histidine tag inserted at its N terminus. To test the functionality of the modified Ded1 protein (hereafter called His6-Ded1p) in S. cerevisiae, the fusion gene under its own promoter was cloned into a yeast centromeric plasmid (YCP, low copy number). The resulting plasmid (YCplac111-His6-Ded1) or a control plasmid harboring the untagged Ded1 gene (YCplac111-Ded1) was transformed into strain ID2-2A (YCplac33-Ded1). Upon plasmid shuffling on 5-fluoro-orotic acid plates and subsequent restreaking on YPD plates, His6-

1 The abbreviations used are: ss, single-stranded; ds, double-stranded; AMP-PNP, 5′-adenylyl imidodiphosphate; YCP, yeast centromeric plasmid.
DED1 complemented the ded1 null allele to the wild-type extent at all temperatures tested (16, 30, and 37 °C), indicating that the modified Ded1p was functional in vivo (data not shown).

The His6-DED1 gene was then cloned into the T7 promoter, and the resulting plasmid (pET22b-His6-DED1) was introduced into a derivative of the E. coli strain BL21(DE3). The His6-Ded1p was overexpressed after isopropyl-1-thio-β-D-galactopyranoside induction (Fig. 1). Although a small portion was insoluble, most of the His6-Ded1p was found in the soluble fraction of cell extracts. The recombinant protein was purified on a nickel-agarose column and was about 90% pure as judged on a Coomassie Brilliant Blue-stained acrylamide gel. The apparent molecular mass of Ded1p (about 68 kDa) coincides with that estimated by sequence analysis. The identity of the protein was verified by Western blot analysis using anti-polypeptide antibodies (data not shown). The polypeptides that co-eluted are most likely E. coli contaminating proteins rather than degradation products, because they also appeared in eluates of E. coli extracts that harbored pET22b without the DED1 gene.

To analyze the importance of the DEAD motif on Ded1p properties, we constructed a mutated allele in which the highly conserved glutamic acid residue was substituted for an alanine. The DEAD and DEAd motifs are variants of the Walker ATPase B motif and the corresponding aspartic and glutamic acid residues have been shown to participate in ATP hydrolysis (15). The mutated DED1 allele was inserted into YCplac111, which was then transformed into the ID2-2A (DED1-URA3) strain. The transformants were unable to grow on 5-fluoroorotic acid medium, showing that the mutated protein is not functional in vivo. This protein, hereafter called the DAAD mutant, was overexpressed in E. coli and purified as for the wild-type protein. The yield and purity were similar to those obtained with the wild-type; in particular, the same contaminating polypeptides were observed.

Ded1p Has ATP-dependent RNA Helicase Activity—To test whether Ded1p has RNA helicase activity, we examined its ability to displace a partial duplex RNA (called “duplex”), as in Ref. 6. To this end, we constructed a standard substrate consisting of a 68-nt RNA annealed over 14 nucleotides to a shorter radiolabeled 43-nt RNA (Fig. 4A). As shown in Fig. 4B, the duplex migrated much slower than the 43-nt ssRNA (compare lanes 2 and 1). The reactions contained 45 fmol (3 nM) of the RNA duplex and were incubated for 10 min at 37 °C with different amounts of Ded1p. The helicase activity was monitored by determining the amount of 43-nt ssRNA (called “monomer”) released from the duplex. In a reaction mixture containing a large excess of Ded1p, no ATP, no duplex dissociation was detected (lane 2). In the presence of ATP, the amount of monomer increased with increasing concentrations of Ded1p (lanes 3–8), showing that Ded1p has an ATP-dependent helicase activity. Quantification of the duplex and monomer showed that 90% of the duplex was dissociated in presence of 50 nM protein, but dissociation was weaker with lower concentrations (see the legend of Fig. 4). Thus, a 15-fold excess of protein was required for nearly complete dissociation. The reason for this requirement is discussed below.

We also measured the unwinding activity as a function of time and found that in the presence of excess protein (80 nM),
maximal dissociation was reached within 1–2 min (data not shown).

The DAAD mutant, which is deficient for ATPase activity, was unable to dissociate the duplex (Fig. 4B, lanes 9 and 10); moreover, the wild-type protein did not dissociate the duplex when ATP was replaced by AMP-PNP, a nonhydrolyzable analog of ATP (data not shown). These results indicate that helicase activity requires ATP hydrolysis.

**Characterization of the RNA Binding Activity of Ded1p**—To investigate further the unwinding activity of Ded1p and the role played by ATP hydrolysis, we examined whether ATP and ADP influence the binding of Ded1p to RNA. We used a filter binding assay in which RNA-protein complexes are retained by a nitrocellulose membrane and free RNA by a charged nylon membrane (see under “Experimental Procedures”). The substrates used were either a duplex of 55 base pairs containing 5′-ends of only 5 and 9 nucleotides (called complete duplex), the partial duplex substrate used in the helicase assay, or its 43-nt monomer. The ability of Ded1p to bind these species was determined by incubating a fixed amount of RNA (40 fmol, 2 nM) with increasing amounts of Ded1p and measuring the amount of protein-bound RNA in each case. Fig. 5A shows that whereas Ded1p had a marked affinity for the monomer (the majority of the RNA molecules were retained on the filter for Ded1p concentrations above 100 nM, with an average $K_d$ of 20 nM), it bound to the complete duplex extremely weakly, if at all (no more than 1% of the input RNA was retained even at the highest Ded1p concentration used). In contrast, Ded1p could bind to the partial duplex, suggesting that this binding can be partially or totally ascribed to the long 5′-ss extensions of this molecule. However, the percentage of input RNA bound was much lower than with the monomer, particularly at low Ded1p concentrations. Because, even at the highest concentrations used, no more than 35% could be bound, it is difficult to decide whether the majority of the partial duplex molecules bind Ded1p with an extremely weak affinity, or whether only a
subpopulation is responsible for the weak binding.

We also measured the RNA binding capacity of the DAAD mutant protein. As shown in Fig. 5B, the mutant bound to RNA with nearly the same affinity as the wild-type. Thus, ATP hydrolysis is not required for Ded1p binding to RNA. Similar results have been reported for other RNA helicases (30–32).

We then analyzed the effects of nucleotides (ATP and ADP) on the RNA binding properties of the wild-type Ded1 protein (Fig. 6). To avoid ATP hydrolysis and duplex unwinding during the measurements, we used a nonhydrolyzable analog of ATP, AMP-PNP. To check that the binding of AMP-PNP mimics that of ATP, we tested its ability to compete with ATP in the ATPase assay, using subsaturating ATP concentrations (half the determined $K_{m}$). Equimolar and 5- and 10-fold excesses of AMP-PNP with respect to ATP, resulted in 50, 75, and 80% inhibition of the ATPase activity, respectively (not shown). Therefore, AMP-PNP can compete with ATP in the ATPase reaction and thus presumably binds to Ded1p similarly. Now, whereas AMP-PNP had a modest effect on the binding of Ded1p to the monomer (the maximal percentage of bound RNA remained nearly the same, and the apparent $K_{D}$ decreased slightly to 12 nM), it affected much more drastically the binding to the partial duplex. Even at low Ded1p concentrations, the percentage of RNA bound was now quite appreciable, exceeding half the value observed with the monomer, and at high Ded1p concentration, it reached a plateau that was consistently above that observed with the monomer (Fig. 6A). We conclude that AMP-PNP greatly increases the affinity of Ded1p for the partial duplex molecules and possibly also the proportion of these molecules that can be bound. In contrast, ADP had almost no effect on the binding of the protein to either partial duplex or monomer (Fig. 6B). In summary, the affinity of Ded1p for the partial duplex varies markedly depending on which nucleotide is present. These results suggest that hydrolysis of ATP to ADP modulates the relative affinities of Ded1p for ssRNA and dsRNA. As discussed below, this modulation might be responsible for the unwinding activity.

**DISCUSSION**

In this work, we have shown that the putative RNA helicase Ded1p, a DEAD-box protein essential for translation initiation in *S. cerevisiae*, is a bona fide RNA helicase. It possesses an RNA-dependent ATPase activity, an RNA unwinding activity that depends on ATP hydrolysis, and a differential binding to ssRNA and dsRNA that is modulated by ADP and the ATP analog AMP-PNP.

**ATPase Activity**—The ATPase activity of Ded1p is highly stimulated by natural RNAs, such as rRNAs and polyadenylated mRNAs, whereas homopolymers are far less efficient. Among the DEAD-box proteins that have been studied biochemically, only DbpA from *E. coli* shows a strong RNA substrate specificity (33, 34). Other characterized DEAD-box and related proteins either do not show any substrate specificity in the ATPase assay or show a specificity that seems unrelated to their assumed *in vivo* role. For example, mammalian eIF4A, which is required for translation initiation of all mRNAs, is more effectively stimulated by poly(U) than by globin mRNA (35). Similarly, the Upf1 protein (superfamily I helicase family), which is involved in nonsense-mediated mRNA decay in yeast, is stimulated by homopolymers but not by total RNA (36). Thus, the physiological substrates of these proteins cannot always be inferred from their *in vitro* preferences. Nevertheless, the fact that polyadenylated mRNAs and rRNAs are the best stimulators of the ATPase activity of Ded1p suggests that Ded1p may interact with these RNAs *in vivo*, consistent with its general role in mRNA translation (19, 20).

We have obtained a $K_{m}$ value for ATP of around 300 μM, i.e., similar to that reported for other DEAD-box proteins, such as p68, yeast eIF4A, and DbpA. This value does not denote particularly tight ATP binding, but it stands below the cellular ATP level (1–10 mM), indicating that Ded1p can bind and hydrolyze ATP in the cell cytoplasm. The specific activity of Ded1p (turnover number of 340–680 min$^{-1}$) is higher than that obtained for eIF4A (3 min$^{-1}$ for the mammalian factor (37) and 6.8 min$^{-1}$ for the yeast factor (17)), SrmB (1.2 min$^{-1}$) (38), p68 (45 min$^{-1}$) (39), RNA helicase II (1.9 min$^{-1}$) (40), RNA helicase A (54 min$^{-1}$) (41), Prp16p (90 min$^{-1}$) (42), and An3 (6 min$^{-1}$) (43), and it is similar to that of Upf1p (490 min$^{-1}$) (36), DbpA (600 min$^{-1}$) (34) and Prp22p (400 min$^{-1}$) (44). Thus, Ded1p has a higher ATPase activity than many DEAD-box and related proteins for which this activity has been measured.

Although the DAAD mutant is as efficient as the wild-type for binding RNA, its ATPase activity is 60-fold lower, implying that the mutation impairs ATP binding and/or hydrolysis. Interestingly in this respect, mutational analyses performed with other helicases have shown that the conserved aspartic and glutamic acid residues are necessary for ATP hydrolysis but not binding (15, 45, 46). We suggest that, analogously, the DEAD motif of Ded1p is directly implicated in ATP hydrolysis.

**RNA Helicase Activity**—We have shown that purified Ded1p exhibits RNA unwinding activity *in vitro*. Among the 39 DEAD-box and related proteins of *S. cerevisiae*, only 4 have been shown to possess RNA unwinding activity: eIF4A (17); Dbp5p, a DEAD-box involved in poly(A)$^{+}$ RNA export (47); and Prp16p (48) and Prp22p (44, 49), two DEAH-box proteins involved in splicing. Upf1p, a superfamily I protein, has also been shown to possess RNA unwinding activity (36). Whereas Prp16p, Prp22p, and Upf1p are active without other proteins, eIF4A needs the assistance of mouse eIF4B, and Dbp5p seems to
require a still unknown cofactor. In contrast, our observation that Ded1p purified from E. coli can unwind an artificial template on its own indicates that it has an intrinsic unwinding activity.

A Molar Excess of Protein over RNA Is Required for Efficient RNA Unwinding—We have found that high concentrations of Ded1p (≥50 nM, i.e. a large excess with respect to the template) are needed for efficient unwinding. This result raises the possibility that unwinding is not a true enzymatic reaction; for example, Ded1p could passively unwind the duplex simply by occupying the ssRNA appearing due to thermal fluctuations. Such a stoichiometric “dissociating” activity has been attributed to DbpA and CsdA, two DEAD-box proteins of E. coli, which do not require ATP for duplex dissociation (50, 51). However, in the case of Ded1p, ATP is required for the unwinding activity, and AMP-PNP cannot substitute for it; moreover, the DAAD mutant, which binds to ss and duplex RNAs as does the wild-type protein but is deficient for the ATPase activity, is also deficient for unwinding. Thus, ATP hydrolysis is necessary for driving the unwinding reaction, arguing that this reaction is a catalytic rather than a stoichiometric process. In addition, we note that the unwinding reaction only takes place in the presence of ATP, i.e. under conditions where the binding preference of Ded1p for ss compared with duplex RNA is precisely minimal (Fig. 6A).

Why, then, is Ded1p required in such a large excess for efficient unwinding? Although we do not exclude that our Ded1p preparation may not be 100% active, we believe that this requirement reflects the reversibility of the reaction. Indeed, we have observed spontaneous reannealing (25–40%) when denatured duplex was incubated under standard conditions in the absence of Ded1p (results not shown). An excess of Ded1p would then be necessary for maximizing the fraction of the template that binds the protein, thereby increasing the unwinding rate and, ultimately, displacing the reaction toward dissociation. We speculate that, in vitro, smaller amounts of Ded1p may be required if RNA-binding proteins bind to the ssRNA during unwinding, preventing reassociation. Such a role has been attributed to the E. coli ssDNA-binding protein, which has been shown to stimulate the activity of some DNA helicases (e.g. 52, 53).

The requirement for an excess protein over RNA is not unique to Ded1p; rather, it has been observed for most RNA helicases studied so far (e.g. Prp16p (48), Prp22p (44), RNA helicase II (40), Upf1p (36), eIF4A (6, 15), p68 (39)). Among the RNA helicases characterized, only NPH-II (54) and the human RNA helicase A (41) could act in catalytic amounts, being able to dissociate a 10-fold molar excess of RNA duplexes. These helicases may have particularly high turnovers for the unwinding reaction; alternatively, with the particular substrates used, the dissociated strands might fold intramolecularly, preventing their reassociation.

What Could be the Mechanism of Unwinding?—Like most DEAD-box and related proteins, Ded1p requires an ss region for binding to RNA, as shown by its inability to bind to a duplex containing very short tails. Therefore, its affinity for the partial duplex probably reflects mainly binding to the ss flanking regions. In the presence of AMP-PNP, this affinity increases to nearly the same level as for ssRNA, possibly reflecting the fact that Ded1p now binds to the ds part of the duplex or to the ss-ds junction. In the presence of ADP, the affinity of Ded1p becomes again higher for ssRNA. These results suggest that ATP binding and hydrolysis modulate the relative affinities of Ded1p for ssRNA versus dsRNA. Although further mechanistic studies are required to understand the coupling between ATPase and helicase activities, this affinity modulation presumably plays a key role in the unwinding process. We hypothesize that a conformational change of Ded1p occurs upon ATP hydrolysis, leading to a preferential binding to ss and hence to the separation of the two strands. Consistently, DNA helicases, which have been characterized extensively with respect to their DNA binding properties, have been shown to couple binding and hydrolysis of nucleotides to conformational changes that alter their affinity for different forms of DNA, thus driving the reaction (55). In particular, Wong and Lohman (56) have shown that binding of ADP to Rep dimer favors a state that binds preferentially to ssDNA, whereas in the presence of a nonhydrolyzable analog of ATP, both ss and duplex DNA were bound, a situation similar to that observed here. These authors proposed an active “rolling” mechanism, in which the active form of Rep is a dimer with both subunits binding alternatively to duplex and ssDNA at an ssDNA-dsDNA junction. Alternative models for unwinding, which do not necessitate helicase oligomerization, have also been proposed (see Ref. 57).

In contrast to DNA helicases, mechanistic studies with RNA helicases are still at their initial stages. However, Lorsch and Herschlag (37, 58) have shown recently that ATP binding and hydrolysis produce a cycle of conformational changes in mammalian eIF4A that modulates its affinity for ssRNA. Further work is needed to establish whether all DNA and RNA helicases use the same mechanism for coupling ATP hydrolysis and unwinding.

Role of the Ded1p RNA Helicase in Translation Initiation—Translation initiation in eukaryotes involves many RNA-RNA, protein-RNA, and protein-protein interactions that have to be disrupted as translation elongation starts, presumably with the help of RNA helicases. Until now, the only protein involved in translation initiation that has been shown to possess RNA helicase activity, is eIF4A. Although current views propose that eIF4A unwinds secondary structures in 5'-mRNA untranslated regions, its precise function is still elusive. Moreover, yeast eIF4A requires a still unknown cofactor for RNA helicase activity. We show here that Ded1p, another DEAD-box protein that is required for translation initiation in yeast, functions as an RNA helicase in vitro on its own. Its ATPase and RNA helicase activities are necessary for its function in vivo because a mutation that abolishes them leads to lethality. Because DED1 has been isolated as a multicopy suppressor of a mutant of the cap-binding protein eIF4E, Ded1p might be involved in the unwinding of cap-proximal secondary structures, perhaps to facilitate cap recognition. More generally, genetic interactions suggest that Ded1p plays a role at the level of ribosome binding (19). It remains to be determined how its RNA helicase activity participates in this biological function.
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