

RNA Structural Rearrangement via Unwinding and Annealing by the Cyanobacterial RNA Helicase, CrhR*

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Rearrangement of RNA secondary structure is crucial for numerous biological processes. RNA helicases participate in these rearrangements through the unwinding of duplex RNA. We report here that the redox-regulated cyanobacterial RNA helicase, CrhR, is a *bona fide* RNA helicase possessing both RNA-stimulated ATPase and bidirectional ATP-stimulated RNA helicase activity. The processivity of the unwinding reaction appears to be low, because RNA substrates containing duplex regions of 41 bp are not unwound. CrhR also catalyzes the annealing of complementary RNA into intermolecular duplexes. Uniquely and in contrast to other proteins that perform annealing, the CrhR-catalyzed reactions require ATP hydrolysis. Through a combination of the unwinding and annealing activities, CrhR also catalyzes RNA strand exchange resulting in the formation of RNA secondary structures that are too stable to be resolved by helicase activity. RNA strand exchange most probably occurs through the CrhR-dependent formation and resolution of an RNA branch migration structure. Demonstration that another cyanobacterial RNA helicase, CrhC, does not catalyze annealing indicates that this activity is not a general biochemical characteristic of RNA helicases. Biochemically, CrhR resembles RecA and related proteins that catalyze strand exchange and branch migration on DNA substrates, a characteristic that is reflected in the recently reported structural similarities between these proteins. The data indicate the potential for CrhR to catalyze dynamic RNA secondary structure rearrangements through a combination of RNA helicase and annealing activities.

The ability of organisms to rearrange nucleic acid secondary structure is crucial for cellular function and is catalyzed by a diverse range of proteins or protein complexes that facilitate nucleic acid annealing and unwinding. Two protein families, nucleic acid-binding proteins and helicases, catalyze these reactions. RNA-binding proteins are structurally unrelated to helicases (1, 2) and rearrange RNA secondary structure through chaperone-mediated annealing or unwinding in ATP-independent reactions (3, 4). Helicases have been classified into five major groups based on characteristic amino acid motifs with the two largest families, superfamilies 1 and 2, composed

of RNA and DNA helicases (5). The other helicase families include proteins possessing fewer conserved motifs and having different substrate specificities.

Biochemically, helicases function as ATP-driven molecular motors, catalyzing NTP-dependent nucleic acid duplex destabilization or strand displacement (6, 7). Although a number of RNA helicases possess RNA unwinding activity *in vitro*, only three have been reported to exhibit intrinsic RNA annealing activity, the highly related yeast nuclear DEAD-box RNA helicases, p68 and p72 (8), and the nucleolar DEXD-box protein, RNA helicase II/Gu (9, 10). Although these helicases unwind dsRNA,¹ the RNA substrates on which they catalyze RNA annealing differ with p68/72 capable of annealing complementary ssRNA into dsRNA, whereas RNA helicase II/Gu forms intramolecular secondary structure in ssRNA (9, 10). Furthermore, whereas unwinding is ATP-dependent (8), annealing is ATP-independent (8, 10). p68/p72 also catalyze RNA strand exchange through the formation and resolution of RNA branch migration structures (8). An alternative mechanism by which RNA structural rearrangements can be effected involves the concerted interaction of an RNA helicase with an RNA-binding protein, examples of which include eIF-4A/B (11) and Brr2/Prp24 (12, 13). These complexes catalyze the interconversion between dsRNA and ssRNA by coupling RNA helicase and annealing activity. Annealing requires the RNA binding domain and not ATP, indicating that the reaction results from chaperone activity (14). These reactions resemble bacteriophage DNA helicases that catalyze DNA strand exchange in cooperation with a ssDNA-binding protein (15).

It has become clear that helicases are related both structurally and mechanistically (1, 2, 16, 17). These similarities extend to the apparently unrelated proteins, RecA and the AAA+ protein, RuvB, involved in the rearrangement of dsDNA secondary structure through a combination of DNA unwinding and annealing, promoting branch migration through Holliday junctions. These structural similarities may also indicate functional similarities, suggesting the potential for other members of the helicase superfamily to both unwind and anneal complementary nucleic acid templates.

In this report, we characterized the biochemical activities of the RNA helicase, CrhR, from the cyanobacterium *Synechocystis* sp. strain PCC 6803. CrhR expression is regulated by the redox status of the electron transport chain with *crhR* transcript accumulating in response to reduction of the electron transport chain between Q_A in photosystem II and Q_O in cyt *b₆f* (18). These components are reduced in response to electron flow derived from either photosynthetic light harvesting or respira-

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¹ The abbreviations used are: dsRNA, double-stranded RNA; DEAD, Asp-Glu-Ala-Asp; ss, single-stranded; IPTG, isopropyl-1-thio-β-D-galactopyranoside; Ni-NTA, nickel-nitrilotriacetic acid; nt, nucleotide(s); ORF, open reading frame; CrhR, cyanobacterial RNA helicase; ATPγS, adenosine 5'-3-O-(thio)triphosphate.

tion of exogenously provided carbon. We proposed that CrhR-catalyzed modulation of RNA secondary structure is involved in the regulation of redox-induced gene expression. Therefore, it was of interest to examine the biochemical activities possessed by CrhR with respect to the structural rearrangement of RNA secondary structure. Characterization of the ability of recombinant CrhR to catalyze these rearrangements indicated that, similar to other RNA helicases, CrhR possesses RNA-stimulated ATPase and bidirectional RNA helicase activity. CrhR also catalyzes annealing of complementary RNA into dsRNA in an ATP-dependent reaction. CrhR rearrangement of RNA secondary structure also extended to RNA strand exchange activity, most probably through the formation and resolution of RNA branch migration complexes. The results indicate that, *in vitro*, CrhR catalyzes complex RNA secondary structure rearrangements through the coupling of helicase and annealing activities, producing RNA structures that are too stable to be resolved by helicase activity alone.

EXPERIMENTAL PROCEDURES

Expression and Purification of Recombinant CrhR RNA Helicase—An N-terminal translational fusion between a His tag and the CrhR ORF was generated by ligation of two DNA fragments coding for *crhR* into BamHI/EcoRI-digested pRSET A (Invitrogen). A 2.1-kbp DraI/EcoRI fragment corresponding to bp 2888247–2890397 of the *Synechocystis* genome (19) coding for the 3′-*crhR* ORF and untranslated region was obtained from an EcoRI fragment containing the entire *crhR* ORF (18). A 5′ fragment comprising bp 2887644–2888246 of the genome was generated by PCR using primers 5′-CGAACGGATCCATGACTA-3′ and 5′-GCGGGATCCGAATTCGGTG-3′. The first primer generated a BamHI site required for the translational fusion, the ATG being the wild type translational start codon for CrhR while the second primer spanned an internal DraI site within the *crhR* ORF (18). Sequencing confirmed the translational fusion and sequence of the 5′-BamHI/DraI region. JM109 transformants were grown in LB-Amp₁₀₀ medium to $A_{600\text{ nm}} = 0.6$ at 37 °C and recombinant protein expression induced by the addition of phage (M13/T7(DE3), 5 plaque-forming unit/cell, Invitrogen) and IPTG (0.5 mM) for 1.5–2 h. To purify the His₆-CrhR recombinant protein, frozen cell pellets were resuspended in one-tenth volume lysis buffer (50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl, 10 mM imidazole) and disrupted by ultrasonication. When indicated, clarified supernatants were treated with RNase (10 μg/ml) at 4 °C for 1 h. The clarified supernatant was passed over a Ni-NTA column, and unbound proteins were removed by extensive washing with lysis buffer containing 50 mM imidazole. Bound proteins were eluted with 75, 125, and 250 mM imidazole in lysis buffer. Protein obtained from the final imidazole elution was stored in 30% glycerol at –80 °C. CrhR remained active under these conditions for at least 1 month. Imidazole did not affect CrhR enzymatic activity. When required, recombinant His₆-CrhC was expressed and purified as described for CrhR (20).

RNA Substrates—ssRNA was produced by run-off transcription from linear plasmids carrying partially complementary DNA inserts using T7 or SP6 RNA polymerase as described by Yu and Owtrim (20). One strand (41 nt for the 5′-tailed substrate and 103 nt for the 3′-tailed substrate) was uniformly labeled with [α -³²P]UTP, whereas the other strand (68 nt for 5′-tailed substrate and 107 nt for the 3′-tailed substrate) was unlabeled. After purification by denaturing urea gel electrophoresis, ~150 pmol of each ssRNA was annealed with an equimolar amount of its non-labeled partial complement in 20 mM HEPES-KOH, pH 7.2, 250 mM NaCl, and 1 mM EDTA by heating to 95 °C for 5 min and slow cooling to room temperature in a heating block. Partially duplexed RNA substrates containing only 5′-ssRNA (see Fig. 2A) or 3′-ssRNA tails (see Fig. 3A) were purified by electrophoresis on 8% native polyacrylamide gels, visualized by autoradiography, and eluted overnight at 4 °C in 0.5 M ammonium acetate, 1 mM EDTA, and 0.1% SDS. For RNA strand exchange assays, the 5′-tailed dsRNA possessing 14 bp of complementary region and an unlabeled 174-nt ssRNA complementary to the labeled strand over 41 nt were used as the substrates. The 174-nt RNA was produced by T7 RNA polymerase transcription from RsaI-cleaved pGEMMO1/2 and purified by denaturing urea gel electrophoresis.

ATPase Assays—ATPase activities were continuously monitored using a coupled spectrophotometric assay (21). The assay uses pyruvate kinase and lactate dehydrogenase to link ATP hydrolysis to NADH oxidation, resulting in a reduction in absorbance at 338 nm. Unless otherwise stated, ATPase assays were performed at 37 °C in a 0.4-ml

reaction volume containing 20 mM HEPES-KOH, pH 7.5, 80 mM KCl, 3 mM Mg acetate, 1 mM dithiothreitol, 5 mM ATP, 2 mM phosphoenolpyruvate, 0.3 mM NADH, 10 μg of poly(A) RNA, 3 units/ml pyruvate kinase and lactate dehydrogenase, and 0.2 μg of purified recombinant CrhR. Reactions were performed in duplicate in 96-well microtiter plates, and the change in $A_{338\text{ nm}}$ was continuously measured for 10 min using a SpectraMAX Plus spectrophotometer (Molecular Devices). The rate of ATP hydrolysis over the linear portion of the curve was quantified using $6300\text{ M}^{-1}\text{ cm}^{-1}$ for the extinction coefficient of NADH, because the steady-state rate of ATP hydrolysis equals that of NADH oxidation.

RNA Helicase Assay—RNA unwinding assays were performed as described previously (20). Unless otherwise stated, reactions were performed for 15 min at 37 °C in a 20-μl reaction volume containing 20 mM HEPES-KOH, pH 8.3, 3 mM MgCl₂, 1 mM dithiothreitol, 5 mM ATP, 20 units of RNasin (Promega), 500 μg/ml bovine serum albumin, 50 fmol of partially duplexed RNA, and 80 ng of recombinant CrhR. Reactions were quenched by the addition of 5 μl of stop solution (150 mM EDTA, 3% SDS, 0.25% bromophenol blue, 15% Ficoll). Reaction products (10 μl) were separated by electrophoresis on 10% SDS-polyacrylamide gels, and radioactive RNA was visualized by autoradiography. In all of the assays, the reactions denoted by ds and ss contained either dsRNA or ssRNA substrates incubated in complete reaction media minus protein for the entire experiment to control for spontaneous unwinding or annealing of the substrates. When indicated, the percentage of the input dsRNA substrate unwound to ssRNA was calculated using a Molecular Dynamics PhosphorImager and ImageQuant software.

RNA Annealing and Strand Exchange Assays—RNA annealing assays were performed exactly as described for the helicase assays with the exception that two complementary ssRNAs were utilized as the substrate and the reaction was performed at pH 7.0. The ssRNA substrates were obtained by heat denaturation of either the 5′- or 3′-dsRNAs for 5 min at 90 °C followed by snap-cooling on ice. Strand exchange assays also corresponded to the RNA helicase reactions with the exception that 80 fmol of a third unlabeled 174-nt ssRNA strand, partially complementary to the radiolabeled strand of the 5′-tailed dsRNA, was included in the reaction. Strand exchange products containing the 174-nt RNA strand will generate a partially duplexed RNA containing 41 bp of duplexed region (Fig. 5A, 41-bp RNA).

Western Blot Analysis—Chemiluminescence was used to detect CrhR in the indicated *Escherichia coli* extracts after separation on 10% SDS-PAGE and transfer to Hybond ECL nitrocellulose as described previously (22). Anti-CrhR (18) and secondary antibodies (Sigma) were used at dilutions of 1:5000 and 1:20,000, respectively.

RESULTS

Expression and Purification of Recombinant His-tagged CrhR RNA Helicase—The CrhR open reading frame was cloned as a recombinant fusion protein containing a N-terminal His₆ tag expressed in *E. coli* JM109 following IPTG induction and affinity-purified over a Ni-NTA column (Fig. 1A, lanes 3–5). A single polypeptide of the expected molecular weight was observed by Coomassie Blue staining. The identity of the recombinant protein was verified as CrhR by Western blot (Fig. 1B) and liquid chromatography/mass spectrometry/mass spectrometry analysis (data not shown).

ATPase Activity of CrhR RNA Helicase Is Stimulated by ssRNA and dsRNA but Not DNA—The RNA dependence of CrhR-catalyzed ATPase activity was determined by coupling ATP hydrolysis with NADH oxidation, the slope being proportional to the rate of ATP hydrolysis. The effects of a representative range of additions to the assay are shown in Table I. CrhR specifically catalyzed ATP hydrolysis as immunodepletion of CrhR effectively abolished the activity. A low level of activity, 2–3-fold above that present in the boiled CrhR control, was observed in the presence of RNase (+ RNase), suggesting that CrhR possesses a low level of intrinsic ATPase activity that is RNA-independent, similar to that reported for p68 (23). However, the reaction was RNA-stimulated as activity was stimulated 6–12-fold by a variety of RNAs. Unexpectedly, ATPase activity was detected in the absence of exogenous RNA, a level that was reduced to background by treatment of *E. coli* extracts with RNase before affinity purification (– RNA). Thus, CrhR-catalyzed ATPase activity differs from other helicases in that

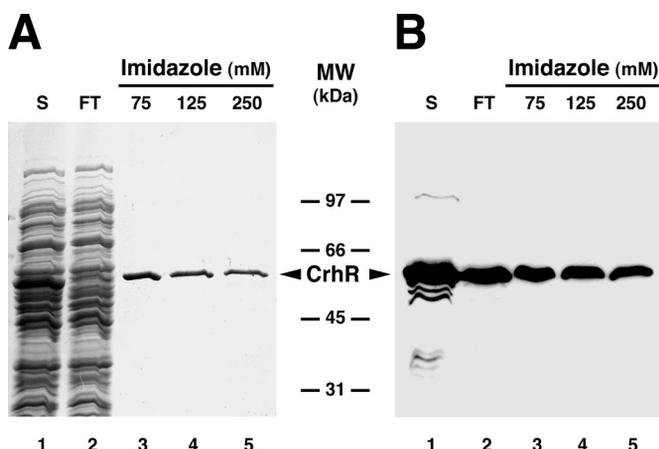


FIG. 1. Expression and purification of recombinant His-CrhR. A, Coomassie Brilliant Blue-stained SDS-PAGE of *E. coli* cell lysates after IPTG induction (S, 30 μ g; lane 1), flow-through from a Ni-NTA column (FT, 30 μ g; lane 2), and elution fractions obtained with the indicated imidazole concentrations (0.5 μ g, lanes 3, 4, and 5). B, CrhR detection by Western blot analysis performed on an SDS-PAGE gel run in parallel with that shown in A. Lane contents were identical to those indicated in A. MW, protein size markers.

E. coli RNA, which remains associated with CrhR during purification, stimulates the activity. In contrast, none of the DNA substrates significantly promoted activity, the low level observed presumably resulting from contaminating RNA. In fact, the significant reduction in ATPase activity in the absence of RNase treatment suggests that DNA acts as a protein trap. ATP and dATP were the only nucleotides that stimulated ATPase activity. K_m values of 0.58 mM, 0.36 mM, and 0.14 μ M were obtained for ATP, Mg^{2+} , and poly(A), respectively. The V_{max} values ranged from 7.9 to 10.0 μ mol of ATP/min/mg protein, corresponding to an ATP turnover number of 452–573 μ mol of ATP/min/ μ mol protein. Therefore, CrhR exhibits RNA-stimulated ATPase activity characteristic of RNA helicases.

Recombinant CrhR RNA Helicase Catalyzes ATP-dependent dsRNA Unwinding in the 5' \rightarrow 3' Direction—To determine the ability of CrhR to unwind dsRNA in the 5' to 3' direction, an artificial RNA substrate containing only 5'-ssRNA tails and an internal 14-bp duplex region was constructed (Fig. 2A, 14-bp RNA) (20). The unwinding of this substrate can only occur if CrhR is moving through the duplex region in the 5' to 3' direction. Recombinant CrhR unwound this dsRNA substrate progressively with respect to time at a constant CrhR concentration as indicated by quantification of the percentage of dsRNA unwound to ssRNA at each time point (Fig. 2B, insert). Unexpectedly, CrhR did not unwind this RNA substrate in a linear manner in response to alterations in either protein or ATP concentration (Fig. 2, C and D). In both cases, unwinding initially increased and then decreased. Reversal of the reaction may result from either a reduction in free Mg^{2+} caused by elevated ATP concentrations or RNA annealing activity. The RNA species migrating with a mobility identical to that of the dsRNA substrate was sensitive to heat denaturation, indicating that it did not represent an irreversibly altered RNA. ATP significantly enhanced the unwinding reaction, indicating that CrhR has an ATP-dependent helicase activity (Fig. 2D). As observed with the ATPase reaction, an examination of the nucleotide requirements for unwinding revealed a strong dependence on adenosine nucleotides with reduced activity observed with the other deoxynucleotides or ribonucleotides tested (Fig. 2E). ATP hydrolysis and not simply ATP binding was required for the reaction, as the slowly hydrolyzable ATP analogue ATP γ S (Sigma) competitively inhibited the reaction in the presence of ATP (data not shown). Evidence that CrhR

catalyzed unwinding was provided by the observation that progressive removal of CrhR by immunoprecipitation resulted in a corresponding reduction in unwinding activity (Fig. 2F). In addition, unwinding was not catalyzed by nonspecific protein, because each reaction contained bovine serum albumin (10 μ g).

CrhR Possesses ATP-stimulated 3'-5'-dsRNA Unwinding Activity—To determine whether CrhR unwinds RNA substrates in the 3' to 5' direction, an artificial partially duplexed RNA containing only 3'-ssRNA tails and an internal complementary region of 25 bp were constructed (Fig. 3A, 25-bp RNA) (20). CrhR unwound this substrate with respect to time and protein concentration in an ATP-stimulated reaction, having kinetics similar to those observed with the 5'-tailed substrate (Fig. 3, B–D). Again, unwinding initially increased and then decreased as the concentration of ATP increased (Fig. 3D). Investigation of the nucleotide requirements for 3'-tailed dsRNA unwinding indicated that ATP most efficiently enhanced the reaction, whereas the other nucleotides were less effective (data not shown).

Recombinant CrhR RNA Helicase Anneals ssRNA into dsRNA—In the helicase assays, progressive decreases in detectable unwinding activity were observed, suggesting that CrhR possessed the capacity to anneal complementary ssRNA strands. Therefore, annealing reactions were performed similar to helicase assays with the exception that the RNA substrate consisted of complementary ssRNAs obtained by heat denaturation of either the 5'- or 3'-tailed dsRNAs (see Figs. 2A and 3A) and the reactions were performed at pH 7.0. Indeed, CrhR-dependent annealing of both pairs of complementary RNAs was observed with the results for the 5'- and 3'-tailed substrates being essentially identical (Fig. 4, A and B). At reduced CrhR concentration, the formation of an RNA species having a mobility identical to that of the 5'- or 3'-dsRNA was observed, presumably as a result of annealing of the complementary ssRNA substrates (Fig. 4, A and B). As CrhR protein concentration increased, the extent of RNA annealing decreased until only ssRNA substrate was present, presumably as a result of helicase activity. Significant levels of spontaneous annealing of either complementary ssRNA pair were not observed in control reactions in the absence of either CrhR or ATP (Fig. 4, A and B, lanes 2, 9, and 10). In these experiments, annealing activity appeared to be protein concentration-dependent with annealing and helicase activity predominating at low and high protein concentrations, respectively. Annealing kinetics were complex, because annealing was also observed at elevated protein and ATP concentrations in Fig. 2, C and D, when the concentration of ssRNA templates was also elevated. A potential explanation for the apparent differences resides in the RNA substrates present in Figs. 2 and 4, with only dsRNA or ssRNA, respectively, creating different dsRNA:ssRNA ratios. Clearly, in all of the experiments, there was a narrow window between which annealing or helicase activity was catalyzed by CrhR.

An important question was whether the annealing reaction was ATP-independent. In contrast to the previously reported cases of RNA annealing, CrhR-catalyzed annealing was ATP-dependent because dsRNA products were not observed in reactions lacking ATP (Fig. 4, A and B, lanes 9 and 10). Informatively, we frequently observed an RNA species of reduced mobility after heat denaturation of the 3'-substrate (Fig. 4B, lanes ss, 3–7, and 9). This RNA apparently corresponds to a partial duplex created by spontaneous intramolecular base pairing within the radioactive ssRNA after denaturation. The inability to detect this RNA at elevated CrhR concentration suggests that CrhR destabilizes this intramolecular structure with the unwinding of this RNA species only occurring under

TABLE I
Influence of various components on CrhR ATPase activity

Initial rates of ATPase activity were determined from the rate of NADH oxidation by continuous spectrophotometric measurement of the absorbance at 338 nm in a standard ATPase reaction containing 200 ng (3.5 pmol) of recombinant CrhR, poly(A) RNA, and ATP. Values are corrected for background oxidation in the presence of boiled CrhR (18.1 and 30.7 $\mu\text{mol}/\text{min}/\mu\text{mol}$ for non-RNased and RNased CrhR, respectively). RNased CrhR and non-RNased CrhR represent recombinant CrhR that was or was not treated with RNase prior to affinity purification, respectively. When specified, poly(A) RNA and ATP were replaced by the indicated RNA and NTP at final concentrations of 25 $\mu\text{g}/\text{ml}$ and 5 mM, respectively.

ATPase activity			ATPase activity		
Addition	Non-RNased CrhR	RNased CrhR	Addition	Non-RNased CrhR	RNased CrhR
$\mu\text{mol}/\text{min}/\mu\text{mol}$ CrhR			$\mu\text{mol}/\text{min}/\mu\text{mol}$ CrhR		
Immunodepleted CrhR	59.7 \pm 0.5	34.4 \pm 4.9	ATP	468.4 \pm 48.8	475.6 \pm 27.1
+ RNase	38.0 \pm 4.0	30.7 \pm 6.5	dATP	441.2 \pm 1.8	464.8 \pm 43.4
- RNA	245.9 \pm 4.7	41.6 \pm 4.2	GTP	12.7 \pm 0.7	0.1 \pm 1.6
<i>Synechocystis</i> Total RNA	398.0 \pm 9.6	177.2 \pm 21.7	<i>Synechocystis</i> genomic DNA	75.9 \pm 41.6	47.1 \pm 5.8
Yeast tRNA	240.5 \pm 2.4	256.8 \pm 34.4	Poly(dA)	63.3 \pm 21.7	57.9 \pm 25.3

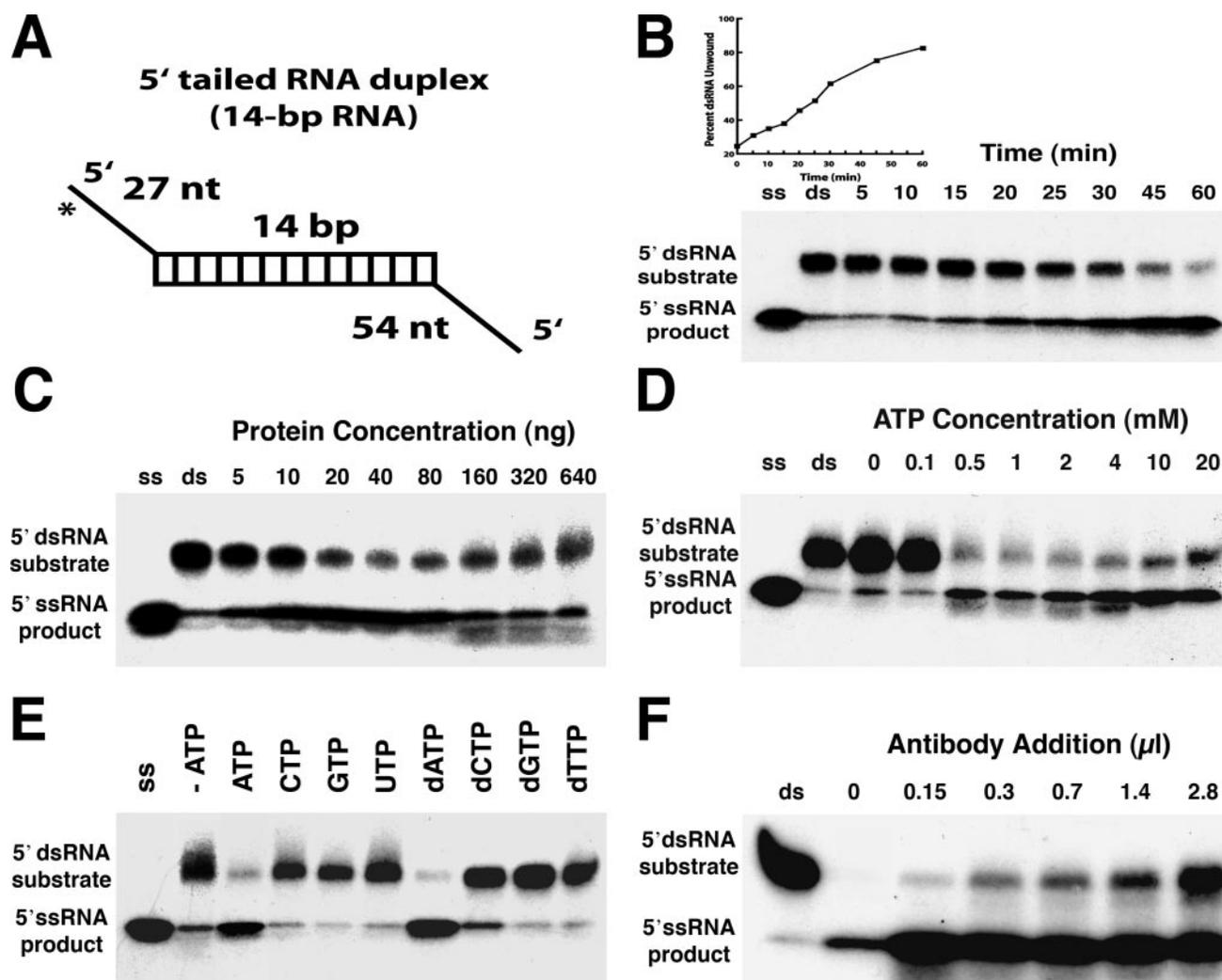


FIG. 2. CrhR possesses ATP-dependent 5' to 3' dsRNA unwinding activity. A, schematic of the artificial dsRNA target possessing only 5'-ssRNA tails (14-bp RNA). The asterisk indicates the ^{32}P -labeled strand. Helicase activity, indicated by conversion of the 5'-dsRNA substrate (ds) to the 5'-ssRNA product (ss) by CrhR was determined with respect to increases in time (40 ng of CrhR) (B), protein (C), and ATP (80 ng of CrhR) (D) concentration. Note the reversal in the efficiency of unwinding at higher protein and ATP concentrations. The graph insert in B indicates the percentage of the total dsRNA substrate that has been unwound to ssRNA obtained by phosphorimaging analysis of the data presented. E, the nucleotide dependence of the unwinding reaction was tested in the presence of 3 mM of the indicated nucleotides. F, RNA unwinding is inhibited by incubation with anti-CrhR antibody. The addition of increasing amounts of polyclonal anti-CrhR antibody (18) to helicase assays containing 80 ng of recombinant His-CrhR progressively inhibited the unwinding activity.

conditions that do not promote annealing (Fig. 4B, lanes 7 and 8). This RNA would be expected to be a product of CrhR-catalyzed unwinding, and low levels are also observed during unwinding (Fig. 3, B–D). The lack of this product at high CrhR concentrations (80 ng) in the absence of ATP suggests that intramolecular unwinding can proceed via an ATP-independ-

ent mechanism (Fig. 4B, compare lane 9 with 10). Thus, CrhR appears to also unwind intramolecular RNA secondary structure under appropriate conditions, potentially through chaperone activity at an elevated protein concentration. The ability of CrhR to catalyze this activity remains to be proven. In contrast, ATP hydrolysis and not solely ATP binding was required for

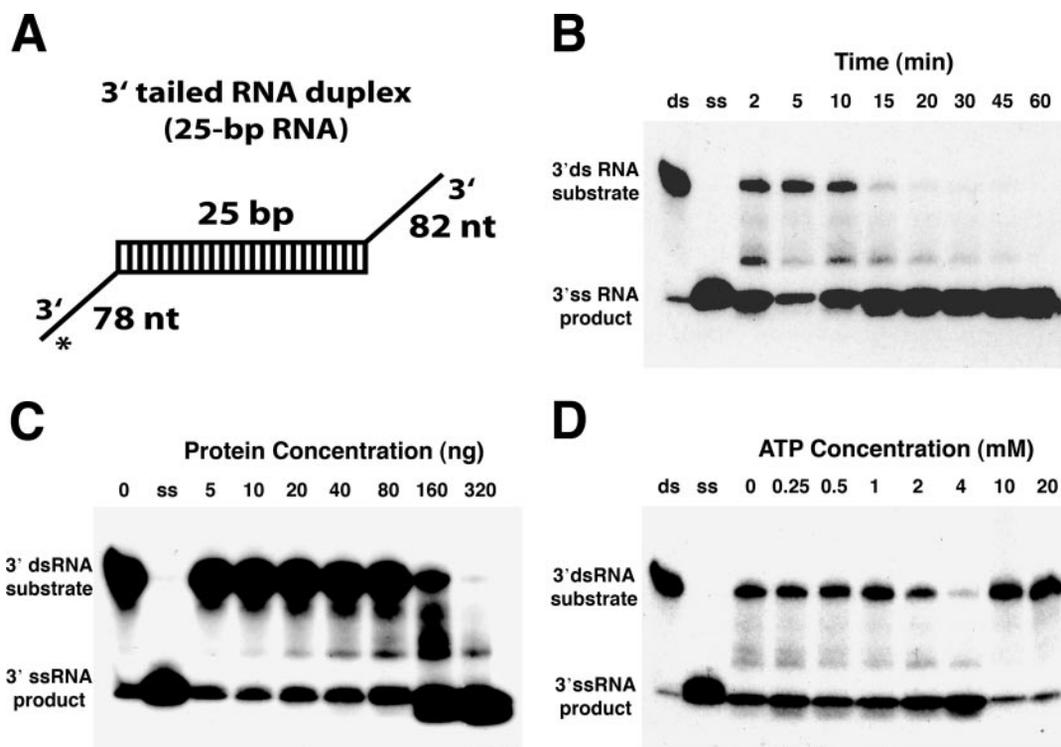


FIG. 3. CrhR unwinding activity on 3'-tailed dsRNA substrates. A, schematic of the artificial dsRNA target possessing only 3'-ssRNA tails (25-bp RNA). The asterisk indicates the ^{32}P -labeled strand. Helicase activity, measured as the conversion from the 3'-dsRNA substrate (ds) to the 3'-ssRNA product (ss) by CrhR was determined with respect to increases in time (300 ng of CrhR) (B), protein (C), and ATP (300 ng of CrhR) (D) concentration.

annealing because duplex RNA was not observed when the slowly hydrolyzable analogue ATP γ S was utilized as the nucleotide source (Fig. 4C, compare lanes 2–5 with 6–9). These data were corroborated by reduced annealing in competition assays containing both ATP and ATP γ S (data not shown). Note that the observed change in mobility of the RNA species does not result from an RNA gel shift, because the reaction products are separated in the presence of SDS. Annealing is also not catalyzed by nonspecific protein, because bovine serum albumin is present in each reaction. Therefore, CrhR possesses both ATP-stimulated RNA helicase and annealing activities.

The results raise the question as to the generality of RNA helicase ability to anneal RNA. The ability of a second recombinant His-tagged cyanobacterial RNA helicase, CrhC, to catalyze the annealing reaction was tested under identical conditions. CrhC is a well characterized RNA helicase that efficiently unwinds the 5'-dsRNA substrate (20). Annealing was tested over a range of pH values since we observed that the annealing reaction proceeded more efficiently at neutral pH than at pH 8.3 used in the helicase assays (Fig. 4D, lanes 3–5). In contrast to CrhR, recombinant CrhC did not catalyze RNA annealing over the pH range (Fig. 4D, lanes 6–8) or protein concentrations (data not shown) tested under conditions identical to those that promote efficient annealing by CrhR. These results also confirm that annealing does not result from contaminating *E. coli* protein(s), because both helicases were purified using identical protocols. Therefore, annealing is not a biochemical activity possessed by all RNA helicases.

Recombinant CrhR RNA Helicase Catalyzes RNA Strand Exchange—The ability of CrhR to catalyze helicase and annealing reactions provides the potential for both reactions to occur concurrently. This was tested by performing an RNA strand exchange assay to assess the ability of CrhR to unwind a dsRNA, replacing one of the duplex strands with a third com-

plementary strand. As shown in Fig. 5A, the reaction contained the 5'-tailed dsRNA (14-bp RNA; 68- + 41-nt strands) plus a 174-nt ssRNA third strand possessing 41 nt complementary to the radioactive strand present in the 5'-tailed dsRNA. The reaction can proceed via two possible pathways with helicase and annealing occurring either concurrently (*top*; Branch Migration path) or sequentially (*bottom path*) (Fig. 5A). CrhR efficiently catalyzed the formation of an RNA species having a lower mobility than the 5'-dsRNA substrate (Fig. 5B). Production of the lower mobility RNA species was CrhR-dependent, because spontaneous formation was not observed in the absence of protein. This reaction product could either represent the 41-bp RNA species (174 + 41 nt) or a branch migration intermediate created by the formation of a complex of the three RNA strands (Fig. 5A). Migration of the strand exchange product with a mobility equal to that of the 3'-tailed dsRNA substrate (whose molecular weight would be comparable with that of the 41-bp RNA) suggested that the product most probably represented the 41-bp RNA. The branch migration intermediate was apparently not observed as a result of the instability of the 14-bp RNA duplex within the 5'-tailed substrate. The lack of the 14-bp RNA is revealing with respect to the reaction mechanism, as one would expect sequential helicase and annealing reactions to produce equivalent levels of the two possible annealed products (14-bp and 41-bp RNA species). This was not the case with the 41-bp RNA product predominating (Fig. 5B), suggesting that the reactions are proceeding concurrently through the production and resolution of an RNA branch migration structure. These reactions were also performed at elevated CrhR concentration in the expectation that the strand exchange reaction would be reversed by the predominance of helicase activity. Production of ssRNA and the concomitant reduction in the 41-bp RNA product were not observed at any protein concentration tested (Fig. 5B). The stability of the 41-bp RNA strand exchange product suggests that CrhR is

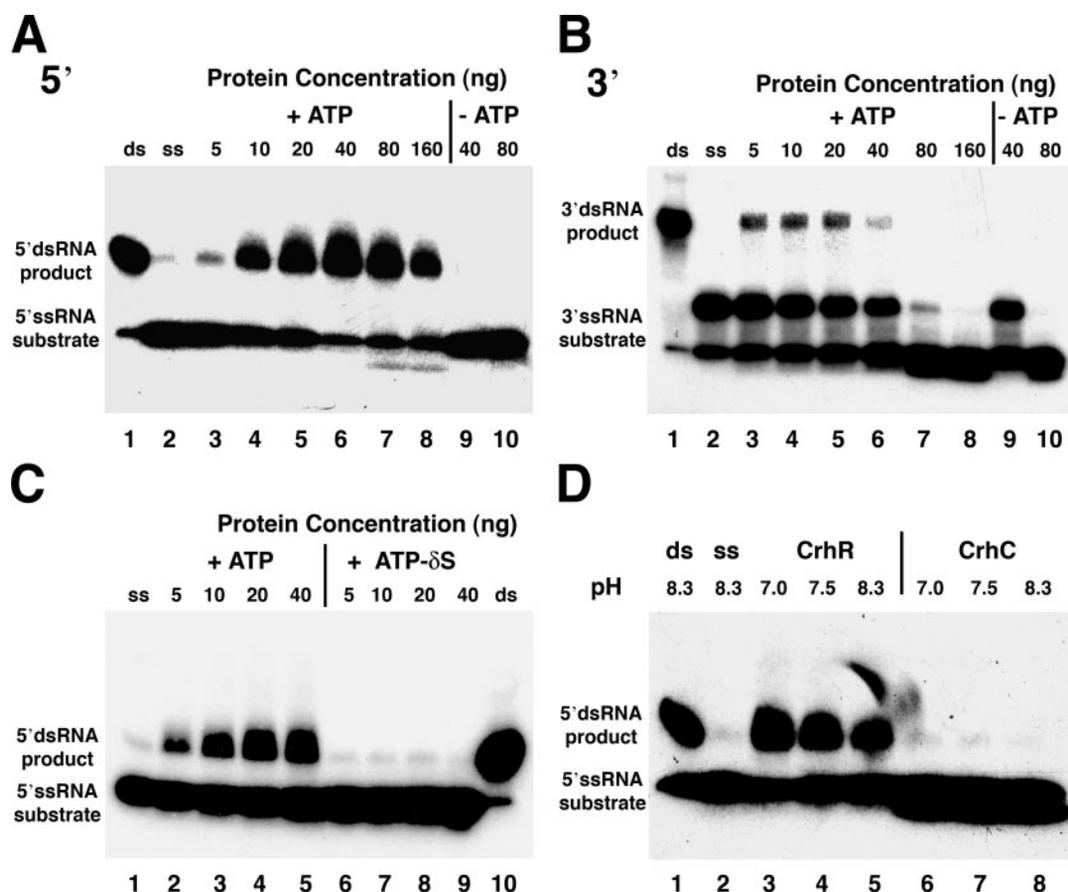


FIG. 4. **CrhR promotes ATP-dependent annealing of complementary ssRNA into dsRNA.** The ability of CrhR to promote annealing of complementary ssRNA into dsRNA was tested with respect to protein concentration using two complementary RNA substrates originating from the 5'-tailed dsRNAs (A) and 3'-tailed dsRNAs (B). The reactions were similar to helicase reactions with the exception that the RNA substrates were ssRNA obtained by boiling the 5'-tailed and 3'-tailed dsRNA substrates shown in Figs. 2A and 3A, and the reactions were performed at pH 7.0. Significant levels of spontaneous annealing were not observed either over the time course of the experiment or over extended periods (data not shown). Note that the 5' and 3' designations do not indicate directionality of annealing in these experiments. C, ATP dependence of annealing. Annealing was performed over a range of CrhR concentrations using either ATP (lanes 3–6) or the slowly hydrolyzable analogue ATP γ S (lanes 7–10). D, pH effects on the ability of 40 ng of either CrhR (lanes 3–5) or a second cyanobacterial RNA helicase, CrhC (20) (lanes 6–8), to catalyze annealing. C and D were performed using the complementary RNA pair obtained from the 5'-tailed dsRNA as substrate.

capable of efficiently and processively catalyzing strand exchange but is unable to unwind the 41-bp RNA duplex present in this reaction product. The combined results suggest that CrhR-catalyzed annealing is more processive than the unwinding reaction.

The RNA strand exchange mechanism was investigated further by determining the formation of the 14-bp and 41-bp RNAs when the reaction was initiated with all three RNA strands in the ss form (Fig. 5C). If annealing alone were occurring, it would be expected that both dsRNA products would form at equal rates. This was not the case, because the CrhR-dependent accumulation of the 41-bp product was significantly lower than that of the 14-bp RNA. Furthermore, the formation of the 41-bp RNA product was only observed when the concentration of the 174-nt strand was approximately equal to that contributed by the denatured dsRNA. Presumably, the 174-nt ssRNA acts as a trap, binding CrhR and inhibiting annealing. This was reflected by an increase in the 14-bp RNA as the 174-nt ssRNA decreased. The trap effect, however, cannot explain the lag in the formation of the 41-bp RNA. These observations are in contrast to Fig. 5B in which the 41-bp RNA formed rapidly in the presence of dsRNA. The formation of the 14-bp RNA and its subsequent interaction with the 174-nt strand appears to be required for the production of the 41-bp RNA product. The results suggest that CrhR preferentially interacts with dsRNA, the substrate that is required to initiate unwinding and allow the concurrent annealing of complementary RNA.

Recombinant CrhR RNA Helicase Unwinds dsRNA in Single Turnover Reactions—The results suggest that annealing is more processive than unwinding and that nucleic acid can interact with CrhR nonproductively, acting as a trap. To provide qualitative evidence that CrhR functions processively, unwinding reactions were performed under single turnover conditions with respect to the RNA substrate. The reaction involved assembly of a CrhR-dsRNA complex in the absence of divalent cation and the presence of ATP, conditions favoring CrhR binding to RNA but not helicase activity (data not shown). Unwinding activity is initiated by the simultaneous addition of divalent cation and an excess of the nucleic acid trap. Helicase activity will be observed only if CrhR unwinding is processive with the helicase remaining bound to the RNA substrate until strand separation is complete (24). Helicase dissociation after a single unwinding step will not produce ssRNA, because the released helicase will be sequestered by the trap nucleic acid. As shown in Fig. 6, CrhR unwound both the 5'- and 3'-dsRNA substrates in the presence of a range of trap nucleic acids. The extent of unwinding obtained in the absence of trap is shown in lanes 2 and 3. CrhR unwinding of the 5'-substrate was incomplete, presumably because the RNA substrate was not saturated with active CrhR protein before the unwinding reaction was initiated (Fig. 6A, lanes 4–10). The trap reactions were repeated with the 3'-tailed RNA duplex, reducing the dsRNA target (25 fmol) and increasing the CrhR (160 ng) levels (Fig. 6B). Under these conditions, CrhR effi-

FIG. 5. CrhR catalyzes ATP-dependent RNA strand exchange. *A*, the substrate RNAs (14-bp RNA and the 174-nt RNA) and potential reactions involved in a strand exchange mechanism. The reaction may proceed through either an intramolecular reaction via branch migration (*top scenario*) or via an intermolecular reaction in which helicase activity is followed sequentially by annealing (*bottom scenario*). *B*, ATP- and CrhR-dependent strand exchange catalyzed by CrhR. Strand exchange reactions were performed as described for unwinding assays with the exception that a third 174-nt ssRNA (80 fmol) was included in the reaction. This RNA has 41 nt complementary to the radioactive strand of the 5'-tailed RNA. *C*, annealing/strand exchange catalyzed by CrhR from ssRNA. Reactions were performed similar to those described in *B* with the exception that the 5'-tailed dsRNA was heat-denatured before addition to the assay. CrhR (80 ng) and RNA concentration derived from the 5'-tailed substrate (50 fmol) were kept constant, whereas the 174-nt ssRNA was varied from 42 to 1700 fmol.

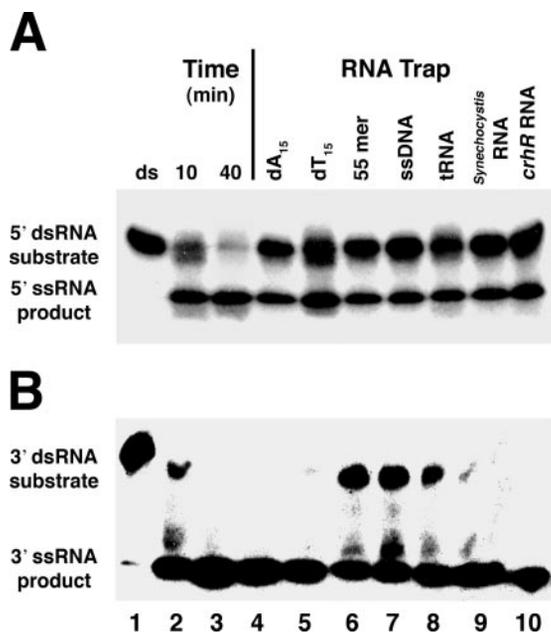
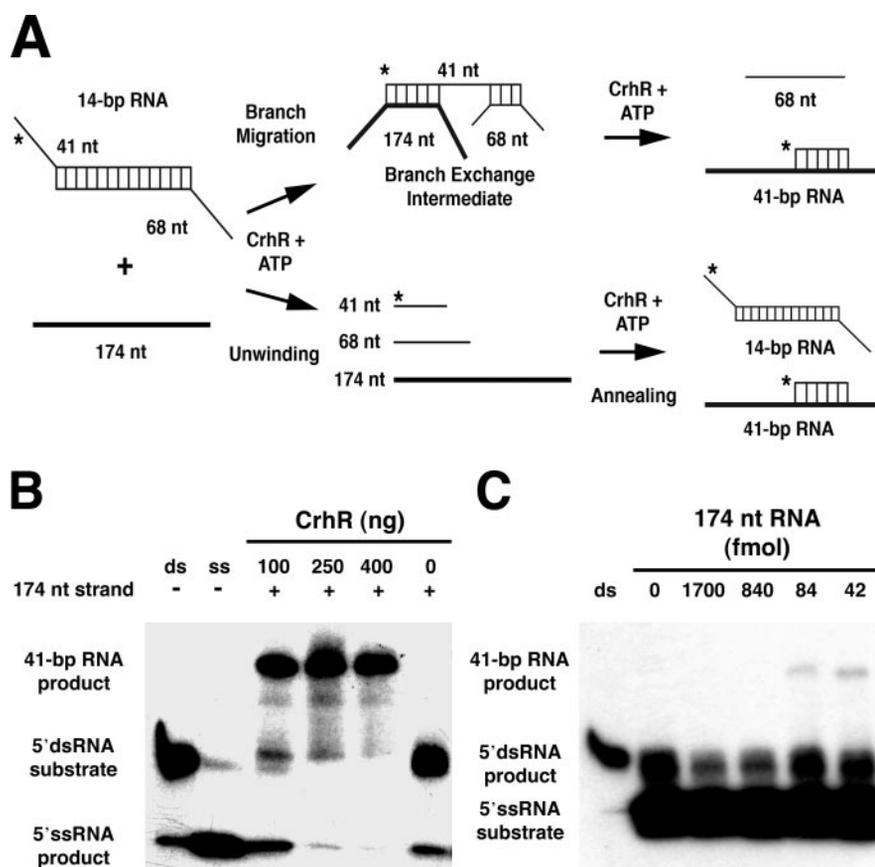


FIG. 6. CrhR unwinds the 5'- and 3'-tailed dsRNA substrates in single turnover assays. Helicase assays were conducted under single turnover conditions that do not allow reinitiation of unwinding once the helicase has dissociated from the initial RNA duplex. Standard helicase assays were performed with 80 ng (*A*) or 160 ng (*B*) of recombinant CrhR for 10 and 40 min (*lanes 2* and *3*). Identical helicase assays were constructed lacking divalent cation and the RNA trap for 10 min at 37°C during which the ATP-dependent loading of CrhR onto the 5'-tailed (*A*) or 3'-tailed RNA duplex substrates (*B*) could occur. Divalent cation and the indicated RNA trap (0.1 μg) were then added to initiate unwinding for an additional 30 min (*lanes 4–10*). Unwinding, indicated by the appearance of the ssRNA product above background levels observed in the absence of protein (*lane 1*), indicates processivity of CrhR through the 14- (*A*) or 25-bp (*B*) duplex regions. Trap RNA sequestering of CrhR released from the first substrate prevents further unwinding.

ciently unwound the 3'-dsRNA target in the presence of a majority of the trap nucleic acids. The trap effect was confirmed since the addition of trap nucleic acid prior to the dsRNA substrate abolished unwinding (data not shown). Based on these results, CrhR unwinding proceeds through 25 bp of dsRNA without dissociating from the substrate.

DISCUSSION

We have shown here that CrhR possesses RNA-stimulated ATPase and bidirectional ATP-stimulated RNA helicase activity, characterizing it as a *bona fide* RNA helicase. In contrast to the majority of characterized RNA helicases, CrhR also catalyzes RNA annealing. In addition, the helicase and annealing reactions can occur concurrently to promote RNA strand exchange most probably through a branch migration mechanism. Therefore, CrhR catalyzes a diverse range of RNA secondary structure rearrangements. RNA annealing does not appear to be a general characteristic of RNA helicases because a second RNA helicase, CrhC (20), did not catalyze this reaction. Therefore, the biochemical properties exhibited by CrhR resemble those possessed by RepA, DnaB, and other replicative DNA helicases, AAA⁺ proteins such as RuvB and also RecA, the latter two proteins required for resolution of Holliday junctions via branch migration. This finding implies that these proteins utilize similar mechanisms to reconfigure nucleic acid secondary structure (24). X-ray crystallographic analysis supports this proposal by revealing that these proteins possess similar three-dimensional conformations (1, 2). It would be interesting to determine whether the RNA annealing activity exhibited by CrhR, p68/72, and RNA helicase II/Gu is manifested in a change to the RNA helicase structural core, providing an indication that these helicases form a subgroup of the DEAD-box RNA helicase family capable of RNA annealing.

CrhR exhibited ATPase and RNA unwinding activity with no apparent RNA substrate specificity, a situation observed for all

RNA helicases (25) with the exception of *E. coli* DbpA (26). The lack of RNA substrate specificity observed *in vitro* raises the question as to how the inappropriate resolution of RNA duplexes is prevented *in vivo*. One mechanism may involve a limit on helicase processivity, thereby preventing unwinding of more stable duplexes (25). CrhR catalyzed the ATPase and unwinding activities characteristic of RNA helicases. The calculated K_m ATP (600 μ M) is higher than that observed for p72 (27) or the bacterial helicases CrhC (20) and DbpA (28) but similar to that reported for yeast eIF4A (29). These values would not be expected to inhibit CrhR activity, because ATP levels do not vary significantly during light-dark transitions (30). The observed specific activity of 500 min^{-1} is at the upper range reported for helicases, similar to DbpA (28) and Ded1p (21). Therefore, CrhR possesses higher ATPase activity *in vitro* than related proteins and has sufficient ATP *in vivo* to actively promote RNA structural rearrangements under either light or dark conditions. CrhR also catalyzed bidirectional strand separation of RNA duplexes up to 25 bp in length but not a 41-bp RNA duplex in the *in vitro* assays used here. Unwinding was also observed under conditions designed to prevent reassociation of CrhR once dissociation from the substrate RNA had occurred. Taken together, these data suggested that CrhR is limited to a maximum of six to seven consecutive unwinding steps, assuming a step-size of 6 as reported for other RNA and DNA helicases (6, 31). The processivity of CrhR-catalyzed unwinding must therefore be considered low compared with the *Vaccinia* virus RNA helicase NPH-II, which can unwind >100 bp of duplex RNA (6). Low helicase processivity may be a reflection of helicase removal from the macromolecular machine in which it functions *in vivo* or a reflection that its physiological function requires the unwinding of only a few base pairs as proposed for eIF-4A (24).

We also show that CrhR anneals complementary RNA, a reaction that has only been demonstrated for the yeast RNA helicases, p68 and p72 (8), and a variety of RNA-binding proteins (3, 4, 13). In addition, CrhR couples the unwinding and annealing activities to effect RNA rearrangements via strand exchange. The reaction produces RNA duplexes whose structure is too stable to be resolved by helicase activity, an indication that annealing is more processive than unwinding. Processive annealing may be required *in vivo*, the stability of the products driving the reaction unidirectionally by preventing helicase activity. Although the mechanism by which CrhR promotes strand exchange remains to be elucidated, the reaction most probably proceeds through a branch migration process, CrhR initially interacting with and subsequently unwinding the duplex RNA while concurrently annealing a complementary RNA. It has been proposed that p68 and 72 also promote RNA strand exchange through a branch migration complex, which represents an enzymatic function other than the isolated combination of the two reactions (8). This was concluded from the observation that p68/p72 branch migration is more processive than its unwinding activity. A similar scenario occurs with CrhR in which strand exchange is more processive than unwinding. CrhR also preferentially initiates strand exchange from a dsRNA substrate, a further indication of a branch migration mechanism.

It is not known which of the observed RNA rearrangements of CrhR catalyzes *in vivo*. It is possible that one or more of the activities observed *in vitro* is peripheral to its physiological role. Association with accessory factors may dictate which functions predominate *in vivo* as proposed for other helicases (24). Although it is clear that the results obtained *in vitro* are produced by a combination of CrhR-catalyzed RNA unwinding and annealing, it is not entirely clear under which conditions one

activity predominates. The conditions that favor either reaction appear to depend on a complex interaction between all of the substrates with the dsRNA:ssRNA ratio being a crucial factor. Similar to CrhR, RNA-binding proteins have a narrow window of activity in which they switch from unwinding to annealing (3).

Intracellular pH may also contribute to the predominating activity, as cyanobacterial cytoplasmic pH decreases from 7.7 to 7.2 in response to a light-dark transition (32). Enhanced annealing at low pH *in vitro* may indicate that the annealing and unwinding reactions may predominate in the dark and light, respectively. This potential divergence of function may be reflected by the pattern of *crhR* expression, which is regulated by light-driven changes in the redox potential of the electron transport chain (18). Although *crhR* transcript accumulates in the light, transcript and protein stability was unexpectedly enhanced in the dark, suggesting that CrhR activity is required under both conditions (18). Whether CrhR unwinds in the light and anneals in the dark remains to be determined. *crhR* expression is also up-regulated by salt (33) and cold stress,² conditions that are known to enhance reduction of the electron transport chain (34). This implies that the active promotion of RNA structural rearrangements by CrhR coupling of helicase and annealing activities is required for cyanobacterial acclimation to a variety of environmental stresses.

What is the fate of the ssRNA product of helicase activity? In the case of DNA helicases, the ssDNA spontaneously adopts the ds form. The situation may differ with RNA helicases in which the annealed RNA structure may be functional. It is well known that the rearrangement of nucleic acid structure during a number of crucial cellular activities involves unwinding and annealing driven by independent proteins (17). The ability of CrhR to perform both functions concurrently provides the ability to rapidly and efficiently convert one ribonucleoprotein structure into another. The *in vivo* role performed by CrhR may therefore involve the alteration of both RNA-RNA and RNA-protein configurations required for adaptation to environmental stress.

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RNA Structural Rearrangement via Unwinding and Annealing by the Cyanobacterial RNA Helicase, CrhR

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