Dissociation of long-chain duplex RNA can occur via strand displacement \textit{in vitro}: biological implications

Matthias Homann*+, Wolfgang Nedbal and Georg Szcakiel*

Forschungsschwerpunkt Angewandte Tumorvirologie, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 242, D-69120 Heidelberg, Germany

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**ABSTRACT**

Hammerhead ribozymes with long antisense flanks (>50 bases) have been used successfully to inhibit replication of human immunodeficiency virus type 1 (HIV-1) in living cells. To explain their increased efficacy versus antisense controls or catalytically inactive derivatives, one can consider dissociation of the ribozyme–product complex to allow a complete catalytic cycle. In this work we investigated the dissociation of a double-stranded RNA with 56 bp \textit{in vitro}. Dissociation was observed in the presence of single-stranded RNA with sequence complementarity to one of the duplex strands. A displacement reaction between RNA single strands and the duplex, but not simple dissociation, was strongly suggested by the concentration dependence of this process, the influence of additional non-complementary sequences on the single strand and by the unusually low Arrhenius activation energy. The strand displacement reaction was slow \textit{in vitro} at 37°C and physiological ionic strength, but was increased to \(k = 10^3 \pm 10^4/M/s\) (∼10⁴-fold) at higher temperatures by cetyltrimethylammonium bromide. This compound is thought to enhance non-sequence-specific association of nucleic acids in a mechanistically similar way to that in which cellular hnRNP proteins are thought to act, indicating that strand displacement can be fast and, more importantly, could be tightly regulated \textit{in vivo}.

**INTRODUCTION**

Dynamic and regulatable interactions between cellular macromolecules are biochemical prerequisites for living cells. Interactions of complementary RNAs play a key role in the regulation of biological processes such as mRNA translation, the splicing of pre-mRNA (1,2) and pathways involving antisense RNA (3). The association of complementary RNA has been described in thermodynamic and kinetic terms in many cases (e.g. 3–7). Kinetic models are appropriate in the case of naturally occurring antisense RNAs, which belong to the best-studied long-chain complementary RNAs (3). The dissociation of RNA duplexes \textit{in vitro} was found to be slow in most cases, depending on the length and base composition of the double strand (8). The rate constants ranged between \(10^{-2}\) and \(10^{-3}/s\) for 10 bp duplexes (9) and even lower values were observed (\(k = 10^{-3}/s\)) for dissociation of cleavage products in the case of short trans-cleaving hammerhead ribozymes forming 16 bp with their target RNA (10). However, little is known about the dissociation of longer RNA duplexes which occur, for example, in the spliceosome (11) or when long hammerhead ribozymes with antisense flanks of 50–280 bases are applied intracellularly (12–14). In the latter case, the dissociation of long duplexes, i.e. the dissociation of the ribozyme–product complex, is a prerequisite for the catalytic turnover of ribozymes, which could explain the observed stronger inhibitory effects versus antisense controls \textit{in vivo} (12,14).

Here we describe the dissociation of a 56 bp duplex RNA in the presence of a displacing single-stranded RNA at physiological ionic strength and temperature. The dissociation rate was dependent on the presence and concentration of the RNA single strand with sequence complementarity to one of the strands forming the duplex. These findings suggest an associative mechanism rather than simple dissociation, i.e. the displacing single strand interacts with the duplex and replaces the homologous strand via formation of a ternary complex. This process could be strongly enhanced in the presence of cetyltrimethylammonium bromide (CTAB), a model compound for cellular facilitator proteins (15).

**MATERIALS AND METHODS**

**RNA synthesis**

RNA was synthesized by run-off transcription \textit{in vitro} from linearized plasmids by T7 RNA polymerase in a 200 µl reaction mixture containing 10 µg DNA template, 18 mM Na₂HPO₄, 2 mM NaH₂PO₄, 8 mM MgCl₂, 20 mM dithiothreitol, 4 mM spermidine, 1 mM each NTP, 5 mM NaCl and 50 U T7 RNA polymerase. For the synthesis of \(\alpha_Y Ȳ9\) the plasmid \(\alpha_Y Ȳ9\) was linearized with \(Bgl\)II, for the synthesis of \(\alpha_Y Ȳ150\) the plasmid \(p_{Z} Y_{150}\) was linearized with \(XhoI\) and for the synthesis of \(S_{R6}\) and \(A_{R6}\) the plasmids \(p_{R-CMV-SR6}\) and \(p_{R-CMV-AR6}\) were linearized with \(NolI\) as described before (16). The ribozyme \(\alpha_Y R_{Z195}\) was transcribed from \(EcoRI\)-linearized plasmid \(p_{Z} Y_{RZ195}\) (17). It contains 195 nt complementary to HIV-1 and 40 non-complementary nucleotides, including a hammerhead ribozyme domain at its 5′-end (17).

*To whom correspondence should be addressed. Tel: +49 6221 424939; Fax: +49 6221 424932; Email: sczakiel@dkfz-heidelberg.de

*Present address: Genzentrum am Max-Planck-Institut für Biochemie, Am Klopferspitz 18a, D-82152 Martinsried, Germany
Figure 1. Schematic depiction of two possible mechanisms of dissociation of an RNA double strand with 56 bp, named ds56. (A) Dissociative mechanism, ds56 may dissociate completely into the sense strand and the radioactively labelled antisense strand. The released sense strand can either re-anneal with the labelled antisense strand, thereby forming the original double strand, or it can bind to a second unlabelled antisense RNA. This unlabelled antisense strand would function as a capture strand for every released sense molecule if it is present in large excess over the double strand, provided that $k_3$ and $k_2$ are in the same range given in this work ($k_{ass} = 10^9$ M$^{-1}$s$^{-1}$). (B) Associative mechanism. Here, the unlabelled antisense strand functions as a displacer strand, as it associates with the double strand to form a ternary intermediate complex characterized by the second order rate constant $k_3$. From this complex, the labelled antisense RNA is displaced. If the association step is rate limiting, then the overall rate depends on the concentration of the displacer strand as well as the duplex.

**Preparation of RNA double strands**

The antisense RNA αY69 (16) was radioactively labelled by incorporation of [α-32P]UTP (250 µCi) during in vitro transcription in 50 µl transcription buffer (see above) lacking unlabelled UTP. Aliquots of 50 ng labelled αY69 were annealed with the complementary RNA SR6 (5 µg) for 5 h in 200 µl buffer containing 100 mM NaCl, 20 mM Tris–HCl, pH 7.4, and 10 mM MgCl$_2$. RNase T1 was added (1000 U, 3 h) to remove single-stranded overhangs. The mixture was phenol extracted five times and the resulting RNA duplex, ds56, was precipitated once in 0.3 M NaOAc, 2.5 vol ethanol and subjected to gel filtration on Sephadex G50. The complete sequence of the antisense strand of ds56 is 5’-AUCUC-CUUGAGGAGUCUCGUCUCGCGGCUCCUCC- CUGCCAUAGGAAGCCUAAG-3’. The 56 double-stranded bases in ds56 are underlined. Due to the fact that RNase T1 cleaves 3’ of G residues, ds56 also contains an AUCU overhang at the 5’-end of the antisense strand (Figs 1 and 2B, ‘as’) and an overhanging C at the 5’-end of the sense strand (Figs 1 and 2B, ‘s’).

**Dissociation kinetics**

The RNA double strand ds56 (0.4 nM) was incubated together with at least four different concentrations of unlabelled single-stranded RNA (e.g. 0.4–5.2 µM AR6) in 100 mM NaCl, 20 mM Tris–HCl, pH 7.4, 10 mM MgCl$_2$ for 6, 12 and 24 h at 37, 47 and 57°C in a volume of 6 µl. The reaction was stopped by the addition of 15 µl stop buffer (7 M urea, 50 mM Tris–HCl, 20 mM EDTA and an additional 0.5% SDS when 1 mM CTAB was used in the dissociation experiment) and the reaction products were analysed on 15% polyacrylamide gels containing 7 M urea. The gels were run at room temperature at 10 V/cm for 12 h in a buffer containing 89 mM Tris, 89 mM borate and 2.5 mM EDTA. The dissociation rates were determined by quantification of the radioactivity in single-stranded and double-stranded RNA bands with a PhosphorImager (Molecular Dynamics). The percentage of released single strand was plotted against time and the individual half-lives ($t_{1/2}$) were determined graphically. $k_{obs}$ can then be calculated from $k_{obs} = \ln(2)/t_{1/2}$. Alternatively, $k_{obs}$ can be derived by fitting plots with the computer program GRAFIT (Erithacus Software, London, UK), giving identical results.

**RESULTS**

The dissociation of two associated complementary RNAs may follow either of two kinetic schemes (Fig. 1): first, a dissociative mechanism, i.e. dissociation of the RNA duplex occurs by melting and separation of the complementary strands (Fig. 1A); second, dissociation can be driven by a displacing third strand with sequence complementarity to one of the two duplex-forming strands via the formation of a ternary complex (Fig. 1B) which, in principle, is similar to some cases of enzyme product release (18). The two mechanisms can be distinguished by kinetic means. The associative mechanism implies that the observed dissociation rate is not dependent on the concentration of the ‘capture’ strand provided that the association step (Fig. 1B, represented by $k_3$) is rate limiting. Under these conditions the kinetics follow a second order reaction and a pseudo first order reaction if either the
displacer strand or the duplex is in large excess. Conversely, the dissociative mechanism implies that the observed dissociation rate is not dependent on the concentration of the capture strand provided that the dissociation step is slower than re-association with the capture strand (Fig. 1A). In this case, dissociation is also independent of biochemical properties of the single strand, such as length and structure.

Dissociation rates are concentration dependent

To distinguish between these two possible mechanisms, we chose the HIV-1-derived antisense RNA αY69 (69 nt) and its complementary target RNA SR6 (645 nt; Fig. 2A), which has been described in terms of structure and association kinetics (16). To synthesize the duplex RNA ds56, αY69 was hybridized with SR6 and the resulting complex was treated with RNase T1 to remove single-stranded overhangs, leaving the double-stranded portion of 56 bp (Fig. 2B). The dissociation of ds56 was investigated in the presence of a large molar excess of the single-stranded RNA AR6 at 57°C (Fig. 3A). We observed a dependence of the rate of the dissociation reaction on the presence and concentration of AR6 (Fig. 3B). Thus, the dissociative mechanism (Fig. 1A) could be ruled out because this concentration dependence can only be explained if the rate limiting step was association between the capture strand AR6 and the released unlabelled single strand. The association rate constant for this pair of RNAs is in the range $1 \times 10^7$/M/s at 37°C and $1 \times 10^6$/M/s at 57°C (data not shown). Thus, at the RNA concentrations used here, the half-lives of the association reaction should be in the range 1–10 s, which is at least $10^5$ times faster than the observed half-lives for dissociation of ds56.

However, the observed concentration dependence is consistent with the associative mechanism shown in Figure 1B if one assumes that the rate limiting step is association between the duplex and the displacer strand, i.e. formation of a ternary complex. In this case, strand displacement follows second order kinetics. If the single strand (AR6) is in a large molar excess over the duplex (ds56), as chosen in this experiment, then the reaction follows pseudo first order kinetics. For each concentration of AR6 the pseudo first order rate constant ($k_{\text{obs}}$) was calculated from the measured half-life $t_{1/2}$ ($k_{\text{obs}} = \ln(2)/t_{1/2}$; Fig. 4A) and a linear correlation between $k_{\text{obs}}$ and the AR6 concentration was found (Fig. 4B). The corresponding second order rate constant was calculated from $k = k_{\text{obs}}/[\text{AR6}]$ to be $k = 3.4$/M/s at 57°C. This rate constant is likely to represent the rate limiting step as proposed by the associative model to be formation of a ternary complex between the duplex and the displacer strand (see Fig. 1B, represented by $k_3$).

Second order reaction kinetics were also observed when the concentration of the duplex ds56 was varied at higher levels (30 nM–1 µM) and the concentration of the displacer αY69 was constant at lower levels (0.5 nM; data not shown).

The associative model implies that the released strand ‘s’ is bound by the displacer strand at the same rate as the complementary strand is released from the duplex ds56 (Fig. 1B). To test this prediction, we performed the experiment schematically depicted in Figure 5A. Here, both strands forming duplex ds56 were 5’-end-labelled and the unlabelled construct αYRz195 (Fig. 2A) was used as displacer. Thus, the fate of both strands of ds56, ‘s’ and ‘as’ respectively, could be followed. In the course of the reaction, αYRz195 displaced the strand ‘as’ and bound to the strand ‘s’ to form a partial duplex RNA that could be separated from ds56 by gel electrophoresis due to its larger size (Fig. 5B). This experiment demonstrates that the displacer strand bound to its complementary strand of the duplex ds56. A quantification showed that release of the single strand ‘as’ (‘displaced strand’ in Fig. 5A) and formation of the complex between αYRz195 and strand ‘s’ occur at indistinguishable rates. These findings further support the associative mechanism. The specific 5’ label of strand ‘as’ shown in Figure 5 is considerably higher than the specific label of strand ‘s’, which is due to a more efficient kinase reaction with the protruding 5’-end of the ‘as’ strand (Fig. 5A).

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Figure 3. Dissociation of the RNA duplex ds56 at 57°C. (A) The overall reaction is release of the labelled antisense strand (as). (B) Autoradiograph of the gel electrophoresis analysis of dissociation of ds56. Lane A, ds56 incubated for 24 h with 5 µM unrelated cut RNA; lane B, ds56 heated to 98°C in stop buffer for 5 min leading to complete denaturation. The arrow on the left points to an unrelated band that does not participate in the reaction. The two upper bands represent the duplex RNA which releases one defined antisense strand after denaturation (lane B). In the neighbouring lanes, time-dependent release of the labelled antisense strand is shown with three time points for each concentration of AR6.

Figure 4. Quantification of dissociation kinetics at 57°C. (A) The percentage of released single strand was plotted against time for all four concentrations of AR6. The observed half-life for each reaction is indicated on the right. (B) Plot of $k_{\text{obs}} (= \ln(2)/t_{1/2})$ against the concentration of AR6 showing a linear correlation. The corresponding second order rate constant $k = 3.4$/M/s was calculated from $k = k_{\text{obs}}/[\text{AR6}]$. 

Figure 5. The overall reaction is release of the labelled antisense strand (as). (A) The percentage of released single strand was plotted against time for all four concentrations of AR6. The observed half-life for each reaction is indicated on the right. (B) Plot of $k_{\text{obs}} (= \ln(2)/t_{1/2})$ against the concentration of AR6 showing a linear correlation. The corresponding second order rate constant $k = k_{\text{obs}}/[\text{AR6}]$ was calculated from $k = 3.4$/M/s at 57°C.
Figure 5. Release of the displaced strand and formation of the duplex, including the displacer strand, occur at the same rate. (A) Schematic depiction of the displacement reaction. Both strands of duplex 1 (ds56) were 5′-32P-labelled using T4 polynucleotide kinase, as indicated by asterisks. In the course of the reaction the ‘as’ strand is released while the ‘s’ strand is captured in duplex 2. (B) Time course of the displacement reaction monitored by non-denaturing polyacrylamide gel electrophoresis.

Dissociation rates are dependent on the length of the displacer strand

A number of single strands that are related to the displacer RNA AR6 and the duplex ds56 respectively have significantly different displacing activities at 37 and 50°C (Table 1). All RNA contain the same sequence stretch of 56 bases contained within the duplex, however, they differ in the length of additional non-complementary sequences (Fig. 2A). Though no simple length dependence is obvious in this experiment, it is noteworthy that longer displacing strands, e.g. αYRz195 or AR6, led to 10- to 100-fold faster reaction rates as compared with the shorter αY150 and αY69. This observation is compatible with the associative mechanism and may indicate an influence of the overall length and/or secondary structure of the displacer strands on interaction with the duplex. In contrast, this observation is not compatible with the dissociative mechanism, since for all displacer strands association with the complementary strand has been measured to be at least 105-fold faster (Homann, unpublished results). Therefore, we conclude that a strand exchange reaction takes place between duplex ds56 and single-stranded AR6 in which formation of a ternary complex is the rate limiting step.

The activation energy of strand exchange

Further mechanistic insight comes from considering the Arrhenius activation energy (Ea) of dissociation. The Arrhenius activation energy of dissociation of RNA double strands with 6–18 bp was determined to be 21–25 kJ/mol/bp, irrespective of the length and base composition of the double strand (5,8,19,20). For dissociation of ds56, Ea was calculated from the temperature dependence of k to be 153 kJ/mol (Fig. 6) or 2.8 kJ/mol/bp. This value is nearly one order of magnitude smaller than expected from an extrapolation of the data obtained for smaller duplexes (5,8,19,20) and, thus, is not compatible with a simple dissociation process.

Regulation of strand displacement

The rate limiting step in the proposed strand displacement reaction is association of the duplex and the displacer strand with a second order rate constant of k = 0.1/M/s at 37°C, which appears to be very small. However, strand displacement is strongly enhanced in the presence of the low molecular weight compound CTAB (21), which is known to mechanistically mimic cellular proteins like hnRNP protein A1 (15). At 1 mM CTAB, the reaction rates of strand displacement between ds56 and AR6 increased by a factor of 7000 at 37°C and 30 000 at 57°C (see table in Fig. 6). This strong enhancement was accompanied by an increased instead of a lowered Arrhenius activation energy (Ea = 215 kJ/mol versus 153 kJ/mol; Fig. 6). A similar observation has been made in the case of the CTAB-mediated increase in the association rates of complementary RNA (Nedbal, Homann and Sczakiel, unpublished data) and the results here are consistent with the assumption that the first step of the associative pathway is enhanced, which is association of the duplex and the single strand to form the ternary complex.
Temperature 645
Second order rate constant $k$ (per M/s) 0.1

The reaction is dependent on the concentration and biochemical properties of the displacer strand, indicating that association of the duplex and the single strand is the rate limiting step. Our kinetic data suggest the formation of a ternary complex and the subsequent displacement of the homologous strand of the duplex. We were unable to isolate the proposed ternary complex, probably due to its fast dissociation. However, one can speculate on the nature of the ternary complex and the molecular mechanism of strand displacement. For example, association of the displacer RNA with the duplex could occur via melting of the duplex ends, described as ‘breathing’ (6,8), followed by invasion of the single strand and subsequent strand displacement. In this respect it is noteworthy that the Arrhenius activation energy of 153 kJ/mol measured for the displacement reaction between ds56 and AR6 corresponds to that the Arrhenius activation energy of 153 kJ/mol measured for subsequent strand displacement. In this respect it is noteworthy that it is formed in an orientation in which the incoming displacer strand is antiparallel with the homologous strand of the duplex. Consequently, it would be parallel with its complementary strand of the duplex (C. Hélène, personal communication), which cannot explain strand exchange.

Alternatively, invasion of the duplex RNA could occur via a loop element of the single strand, as is indicated by the interaction between a GNRA loop and the minor groove of an RNA helix which was observed in a group I intron of the bacteriophage T4 (26,27). However, since a GNRA loop cannot be found in the predicted secondary structures of either of the displacer strands, there is no evidence supporting this model.

The rate constant $k = 0.1$ M/s at 37°C for the displacement reaction seems to be too small to be relevant for cellular reactions. However, many viral (28,29) and cellular proteins (30–32) are known to increase the rate constants for association of nucleic acids by up to three orders of magnitude. In the presence of the facilitator CTAB, the rate constants for strand exchange increased dramatically to $k = 10^3–10^4$/M/s (Fig. 6). This observation supports the model of formation of the ternary complex as rate limiting. Furthermore, the strong CTAB-mediated increase in strand exchange indicates that the reaction can be regulated and could play an important role in vivo.

The results described here may have several biological implications. Firstly, catalytic turnover of ribozymes could be imagined assuming displacement of cleaved substrates of a ribozyme–product complex by an incoming uncleaved substrate in living cells which is compatible with the observed stronger inhibitory effects of long-chain hammerhead ribozymes versus antisense controls (see for example 12–14,17). We tested this possibility with an ‘asymmetric’ ribozyme (17) forming 63 bp with its substrate. In this case, the uncleaved substrate was able to displace the cleaved product with a similar rate as was measured for the strand exchange between ds56 and AR6 (M. Homann, unpublished results). In view of the potential biological and clinical role of trans ribozymes, it may further be of interest to analyse target sequences with respect to the ability to displace cleaved products from the ribozyme–product complex, which could be done by means of in vitro selection techniques. Here, the aim is to identify pairs of ribozyme–product RNAs where the RNA substrate serves as a good displacer and the products are good leaving strands.

One could speculate that an alternative way of formation of a ternary complex involves formation of a partial triple helix between the duplex and the displacer strand. There is a polyuridine stretch in the sense strand of the duplex ds56 and, correspondingly, polyuridyrimidine stretches in both antisense strands (positions 28–42 in ds56). However, if a triple helix was formed, it is likely that it is formed in an orientation in which the incoming displacer strand is antiparallel with the homologous strand of the duplex. Consequently, it would be parallel with its complementary strand of the duplex (C. Hélène, personal communication), which cannot explain strand exchange.

Table 1. Dependence of duplex dissociation on the displacing strand

<table>
<thead>
<tr>
<th>Displacer RNA</th>
<th>Total length (nt)</th>
<th>Temperature (°C)</th>
<th>Second order rate constant $k$ (per M/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AR6</td>
<td>645</td>
<td>37</td>
<td>0.1</td>
</tr>
<tr>
<td>αYRz195</td>
<td>235</td>
<td>37</td>
<td>0.1</td>
</tr>
<tr>
<td>αY150</td>
<td>150</td>
<td>37</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>αY69</td>
<td>69</td>
<td>37</td>
<td>&lt;0.002</td>
</tr>
</tbody>
</table>

*All RNA contain the same 56 bases complementary to the sense strand of ds56 (Fig. 2A and B) plus additional HIV-1-derived as well as vector-derived nucleotides.

**DISCUSSION**

In this work we describe a strand exchange reaction between a 56 bp duplex RNA and RNA single strands with sequence complementarity to one of the duplex strands. In accordance with an associative mechanism for dissociation (Fig. 1B), the reaction is dependent on the concentration and biochemical properties of the displacer strand, indicating that association of the duplex and the single strand is the rate limiting step. Our kinetic data suggest the formation of a ternary complex and the subsequent displacement of the homologous strand of the duplex. We were unable to isolate the proposed ternary complex, probably due to its fast dissociation. However, one can speculate on the nature of the ternary complex and the molecular mechanism of strand displacement. For example, association of the displacer RNA with the duplex could occur via melting of the duplex ends, described as ‘breathing’ (6,8), followed by invasion of the single strand and subsequent strand displacement. In this respect it is noteworthy that the Arrhenius activation energy of 153 kJ/mol measured for the displacement reaction between ds56 and AR6 corresponds to the melting of 7–8 bp in short RNA duplexes (8). A related reaction is known from recA-catalysed strand exchange in homologous recombination (for reviews see 22,23). In this reaction, recA-covered double and single strands are aligned by homologous recombination (for reviews see 22,23). In this case, the uncleaved substrate was able to displace the cleaved product with a similar rate as was measured for the strand exchange between ds56 and AR6 (M. Homann, unpublished results). In view of the potential biological and clinical role of trans ribozymes, it may further be of interest to analyse target sequences with respect to the ability to displace cleaved products from the ribozyme–product complex, which could be done by means of in vitro selection techniques. Here, the aim is to identify pairs of ribozyme–product RNAs where the RNA substrate serves as a good displacer and the products are good leaving strands.

Furthermore, at a more basic level, the energy barrier for RNA strand exchange may be much lower than expected for simple dissociation, indicating that interactions between RNA could be more dynamic than expected and would not necessarily lead helicases or single strand stabilizing proteins. There is, for example, an extensive base pairing interaction between the small nuclear RNAs U4 and U6 in the spliceosome (2). However, the switch from the inactive to the active spliceosome is correlated with displacement of U4 from U6, which then forms base pairs with U2 to a similar extent (11). According to the mechanism described in this work, U2 might play a more active role in displacing U4 from U6. It should be noted, however, that neither
the intracellular concentrations of nucleic acids nor the influence of subcellular localization on RNA–RNA interactions are known.

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