CTAB-mediated enrichment for active forms of novel dimeric maxizymes

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Abstract We demonstrated previously that shortened forms of (stem II-deleted) hammerhead ribozymes with low intrinsic activity form very active dimers with a common stem II (very active short ribozymes capable of forming dimers were designated maxizymes). As a result of such a dimeric structure, heterodimeric maxizymes are potentially capable of cleaving a substrate at two different sites simultaneously. In this case, active heterodimers are in equilibrium with inactive homodimers. Longer forms of common stem II can lead to enrichment of the active heterodimers in vitro. In this study, we investigated whether the cationic detergent CTAB, which is known to enhance strand displacement of nucleic acids, might inhibit the dimerization of maxizymes. Significantly, under all conditions examined, CTAB instead enhanced the activity of a variety of maxizymes, with the extent of enhancement depending on the conditions. The activity of our least stable, least active maxizyme was enhanced 100-fold by CTAB. The strand displacement activity of CTAB thus appears to enhance the conversion of alternative conformations of inactive maxizymes, with intra- and inter-molecular hydrogen bonds, to active forms. Thus, our smallest maxizyme can also be considered a potential candidate for a gene-inactivating agent in vivo, in view of the fact that various facilitators of strand displacement reactions are known to exist in vivo (indeed, a separate experiment in cell culture supported the conclusion that our smallest maxizyme is a good gene-inactivating agent). Although activities of ribozymes in vitro do not necessarily reflect their activities in vivo, our findings suggest that the activity of ribozymes in vivo can be better estimated by running ribozyme kinetics in the presence of CTAB in vitro.

Key words: Hammerhead ribozyme; Maxizyme; Strand displacement; Facilitator; Kinetics; Gene therapy

1. Introduction

The hammerhead ribozyme is one of the smallest RNA enzymes [1–4] (Fig. 1A). Because of its small size and potential utility as an antiviral agent it has been investigated extensively in terms of the mechanism of its action and possible applications in vivo [1–8]. The hammerhead ribozyme was first recognized as the sequence motif responsible for the self-cleavage (*cis* action) of the satellite RNAs of certain viruses [9–11]. The putative consensus sequence required for activity has three duplex stems and a conserved 'core' of two non-helical segments, plus an unpaired nucleotide at the cleavage site. The *trans*-acting hammerhead ribozyme [3] consists of an antisense section (stem I and stem III) and a catalytic domain with a flanking stem/loop II section. Such RNA motifs can cleave RNA targets at specific sites (most effectively at GUC [12–17]).

In attempts to identify functional groups and to elucidate the role of the stem II region, various modifications and deletions have been made in this region [4,18–24]. For the application of such enzymes as therapeutic agents for the treatment of infectious diseases, minimized hammerhead ribozymes (minizymes) seem to be particularly attractive because such removal would obviously reduce the cost of synthesis, and increase the overall yield of the desired polymer and simplify purification. However, the activities of minizymes are two to three orders of magnitude lower than those of the parental hammerhead ribozymes, an observation that led to the suggestion that minizymes might not be suitable as gene-inactivating reagents [23]. Thus, conventional hammerhead ribozymes with a deleted stem II (minizymes) came to be considered crippled structures, attracting minimal interest because of their extremely low activity, as compared to that of the full-sized ribozyme. However, we recently identified very active short ribozymes and we presented evidence that short ribozymes with such high-level activity actually form dimeric structures [4,25] (Fig. 1B). In order to distinguish monomeric forms of conventional minizymes that have extremely low activity from our novel dimers with high-level activity, the latter very active short ribozymes capable of forming dimers are designated 'maxizymes'. We also demonstrated that heterodimeric maxizymes might be potentially useful as gene-inactivating agents since a heterodimer, because of its two independent catalytic cores (Figs. 1C and 2), can cleave a single substrate at two independent sites simultaneously (in contrast to homodimeric maxizymes that cleave at one site only Fig. 1B). In previous reports we described the physical properties of various kinds of homodimeric and heterodimeric maxizymes, and we demonstrated that all heterodimeric maxizymes tested were capable of cleaving the HIV-1 tat mRNA [4,25,26] at two GUC triplets simultaneously (Fig. 2). Simultaneous cleavage resulted in an overall increase in the efficiency of degradation of the target RNA [25,26]. Moreover, we found that the activity of the maxizymes in vitro increased with increases in the length of the common stem II of the dimer [25]. The stability of the dimeric structure depends not only on the number of G-C pairs in the common stem II of the dimeric maxizyme but also on the concentration of Mg^{2+} ions and on whether or not substrate molecules are bound to the dimer [4,25].

Many so-called facilitators have recently been identified that significantly increase the rates of formation of RNA duplexes and of ribozyme-catalyzed reactions [27–38]. These facilitators include nuclear proteins, the HIV-1 nucleocapsid (NC) protein, and cationic detergents. It seems possible, therefore, that the capacity of ribozymes for the rapid and specific

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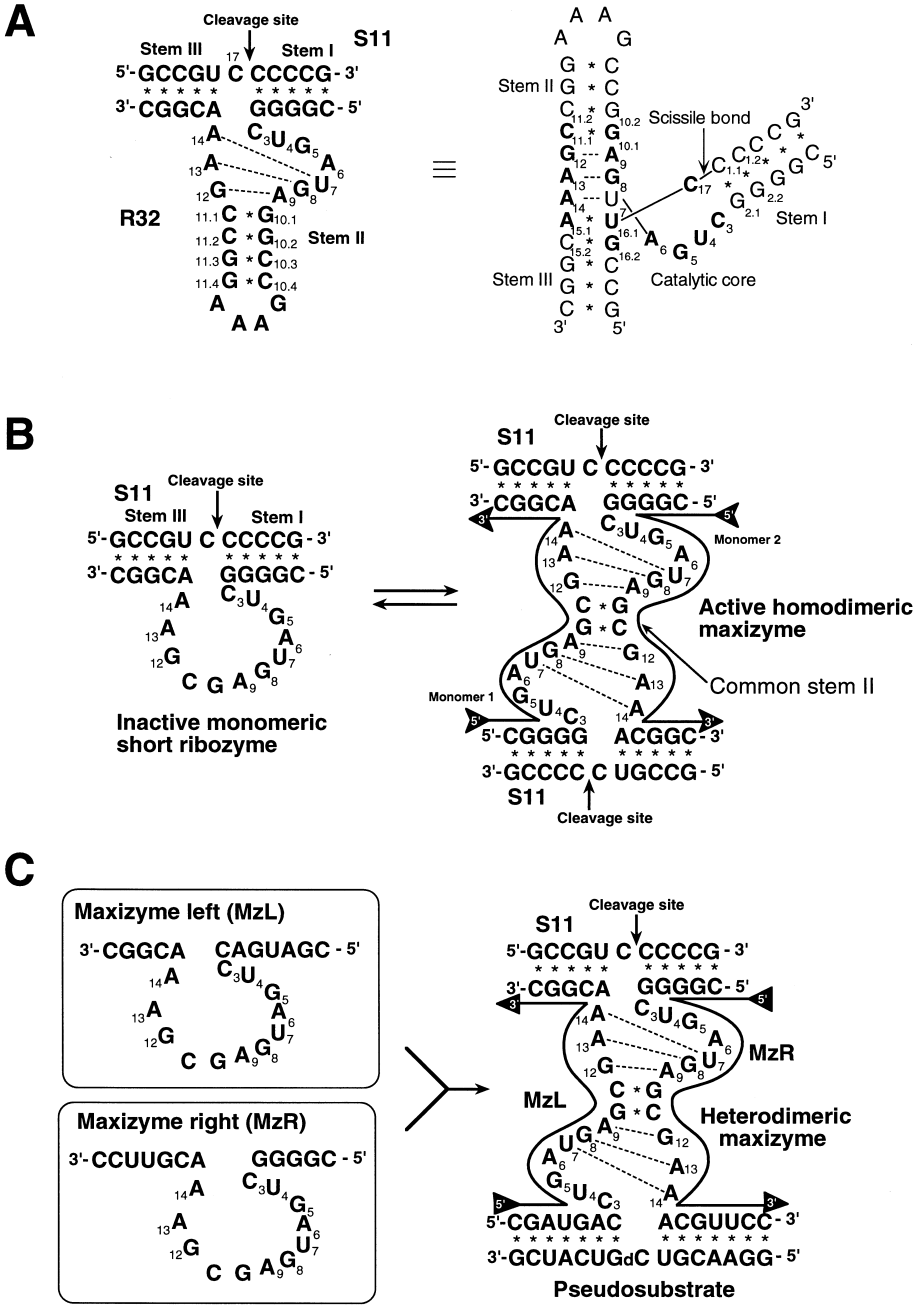

Fig. 1. Secondary structures of (A) the wild-type hammerhead ribozyme (R32), (B) the maxizyme that is capable of forming a homodimer, and (C) the heterodimeric maxizyme. A schematic representation of the overall folding of the hammerhead ribozyme is shown in A on the right. Homodimeric maxizymes have two identical substrate-binding sites, whereas the MzL-MzR heterodimeric maxizyme can generate two different binding sites: one is complementary to the sequence of the substrate (S11), that we used in this study and the other is complementary to a substrate with a different sequence (in this figure, an uncleavable pseudosubstrate is shown). S11 can be cleaved only after the formation of a dimeric maxizyme.

cleavage of RNAs in vivo might be enhanced by such facilitators if ribozymes or substrates in an inactive conformation could be converted to active forms in vivo.

The dimerization of maxizymes is a complicated process. In this case, there is a possibility that the strand displacement activity of CTAB might inhibit the dimerization process of maxizymes. Therefore, in this study, we investigated the effect (stimulatory or inhibitory) of just one cationic detergent, namely CTAB (cetyltrimethylammonium bromide), on the overall activity of maxizymes; one with a long common stem II that can form a stabler and active heterodimer, and the other one with a short common stem II that yields significantly higher proportions of inactive homodimers in vitro (Figs. 1 and 3). We demonstrate that the strand displacement activity of CTAB appears to enhance the conversion of alternative conformations of inactive maxizymes, with intra- and inter-molecular hydrogen bonds, to active forms so that the maxizyme with a short common stem II gains significantly higher activity in vitro in the presence of CTAB. This finding is in agreement with the conclusion based on a separate experiment that the maxizyme with a short common stem II is as active as the maxizyme with a long common stem II in

cultured cells, wherein various facilitators exist that enhance strand displacement reactions similarly to CTAB. This in turn suggests that CTAB might be useful in predicting activities of ribozymes in vivo.

2. Materials and methods

2.1. Synthesis of maxizymes and substrates

Maxizymes and their short substrates Figs. 1 and 3 were synthesized chemically by a DNA/RNA synthesizer (model 394; Perkin Elmer, Applied Biosystems (ABI), Foster City, CA, USA). Reagents for RNA synthesis were purchased from Glen Research (Sterling, VA, USA). Oligonucleotides were purified as described in the user bulletin from ABI (53, 1989) with minor modifications. Further purification was performed by polyacrylamide gel electrophoresis, as described previously [4,25].

2.2. Measurements of kinetic parameters

Measurements of kinetic parameters of reactions catalyzed by maxizymes were made with 5′-^{32}P-labeled short substrates: S19 (5′-CAGAACAGUCAGACUCAUC-3′), which included GUC triplet-2 of 272-meric HIV-1 tat mRNA, was used for the reactions mediated by 2 bp and 5 bp dimeric maxizymes (Fig. 3A,B), and S11 (5′-GCCGUCCCCCG-3′) was used for the reactions mediated by homodimeric and heterodimeric maxizymes (Fig. 1B,C). The terms 2 bp and 5 bp dimeric maxizymes refer to dimeric maxizymes with two and five G-C pairs, respectively, in the common stem II. Reaction rates were measured, in 25 mM $MgCl_2$, 50 mM Tris-HCl (pH 8.0) and 10 mM NaCl under single-turnover conditions at 37°C, in the presence or absence of 50 μM CTAB (Sigma, St. Louis, MO, USA).

Reactions were initiated by the addition of appropriate amounts of $MgCl_2$ after pre-incubation of reaction mixture that contained all components apart from $MgCl_2$ for several minutes at 37°C. Reactions were stopped by the removal of aliquots from the reaction mixture at appropriate intervals and mixing them with an equivalent volume of a solution that contained 100 mM EDTA, 9 M urea, 0.1% xylene cyanol, and 0.1% bromophenol blue. The substrate and the products of the reaction were separated by electrophoresis on a 5%–20% polyacrylamide/7 M urea denaturing gel and were detected by autoradiography. The extent of cleavage was determined by quantitation of radioactivity in the bands of substrate and products with a Bio-Image Analyzer (BAS2000; Fuji Film, Tokyo).

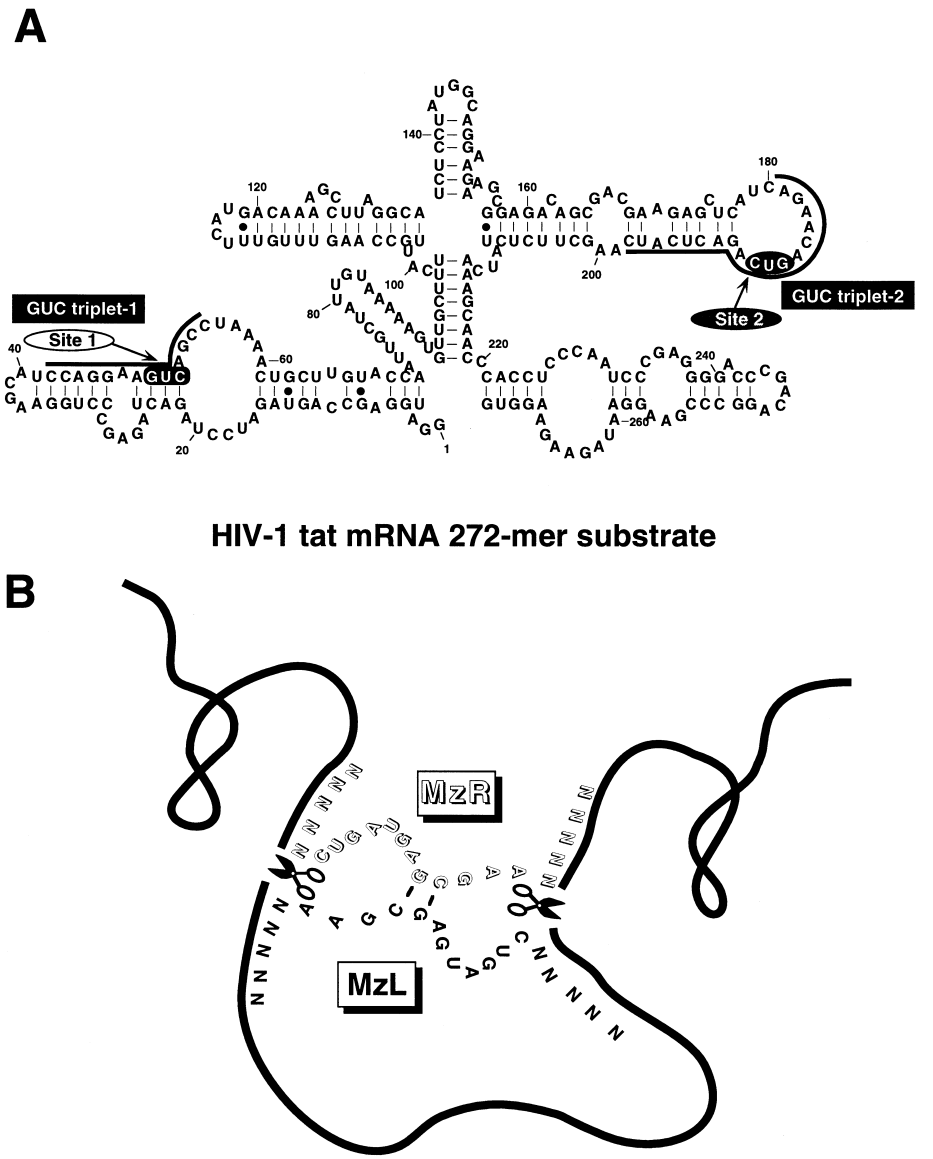

Fig. 2. Simultaneous cleavage of HIV-1 tat mRNA at two sites by a dimeric maxizyme. A: The secondary structure of HIV-1 tat mRNA, as predicted by MulFold (Biocomputing Office, Biology Department, Indiana Univ., IN, USA). Cleavage site 1 (GUC triplet-1) and cleavage site 2 (GUC triplet-2) are indicated by arrows. B: Simultaneous cleavage (scissors) of a target mRNA at two sites by a dimeric maxizyme.

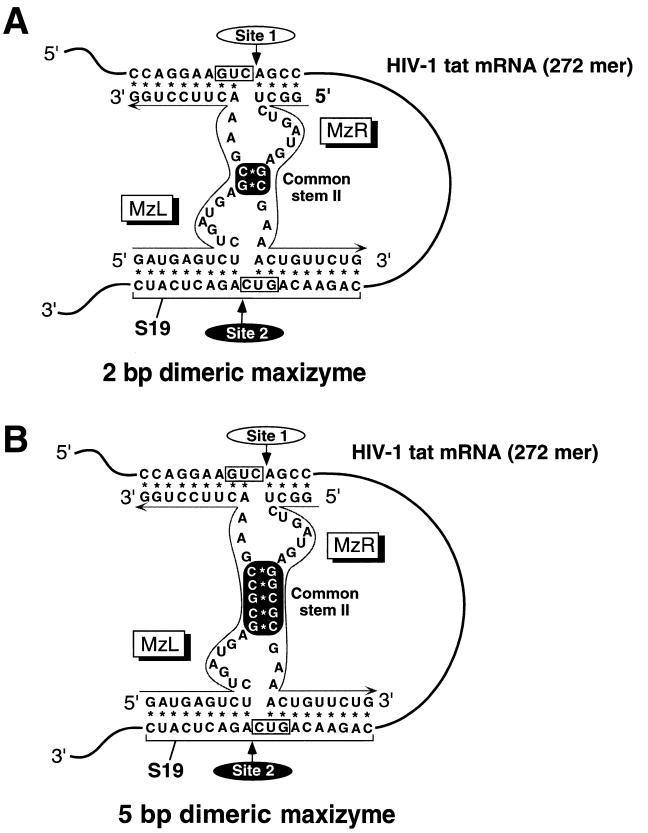

Fig. 3. Secondary structures of dimeric maxizymes that target HIV-1 tat mRNA. A: The 2 bp dimeric maxizyme with two G-C pairs in the common stem II. B: The 5 bp dimeric maxizyme with five G-C pairs in the common stem II. The short substrate of 19 nucleotides (S19), indicated by a long bracket, was used in this study.
←

2.3. Measurement of the melting temperature (T_m) of each dimeric maxizyme

In order to determine the T_m of each maxizyme (2 bp maxizyme left (2 bp MzL), 2 bp maxizyme right (2 bp MzR), 5 bp maxizyme left (5 bp MzL) and 5 bp maxizyme right (5 bp MzR); Fig. 3), we monitored the thermal denaturation of ribozymes with a UV spectrophotometer (model 2100S; Shimadzu, Kyoto). Solutions of maxizymes (2 μM) were prepared in 50 mM Tris-HCl buffer (pH 8.0). After degassing, these samples, without Mg^{2+} ions, were heated at 80°C for 3 min and then slowly cooled to 5°C over the course of 20 min. Then a concentrated solution of Mg^{2+} ions was added to each sample to give a final concentration of $MgCl_2$ of 25 mM. The absorption of the samples at 260 nm was monitored continuously at 5°C for 10 min and then the temperature was raised from 5°C to 80°C at a rate of 1°C/min. The T_m was determined by plotting the derivative of the thermal denaturation curve.

3. Results and discussion

3.1. Kinetic analysis of the reaction catalyzed by a 5 bp dimeric maxizyme targeted to HIV-1 mRNA

Our previous analysis indicated that increases in the length of the common stem II are associated with increases in the

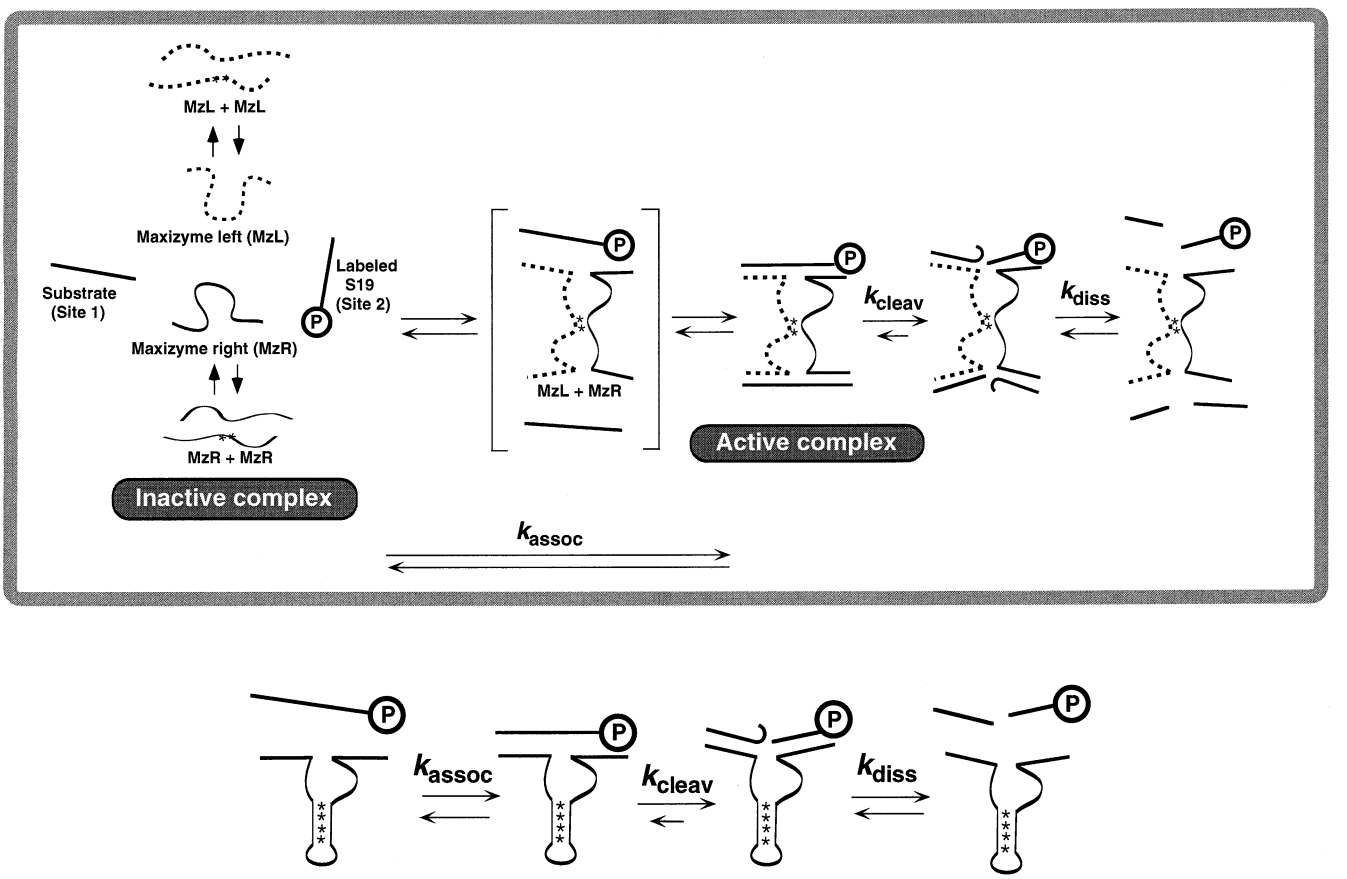

Fig. 4. Reaction scheme of the cleavage mediated by dimeric maxizymes and hammerhead ribozymes. The upper panel shows the reaction scheme for dimeric maxizymes and the lower panel shows that for the parental hammerhead ribozyme. The dimeric maxizymes are more likely to exist in their inactive conformations than in their active conformations.

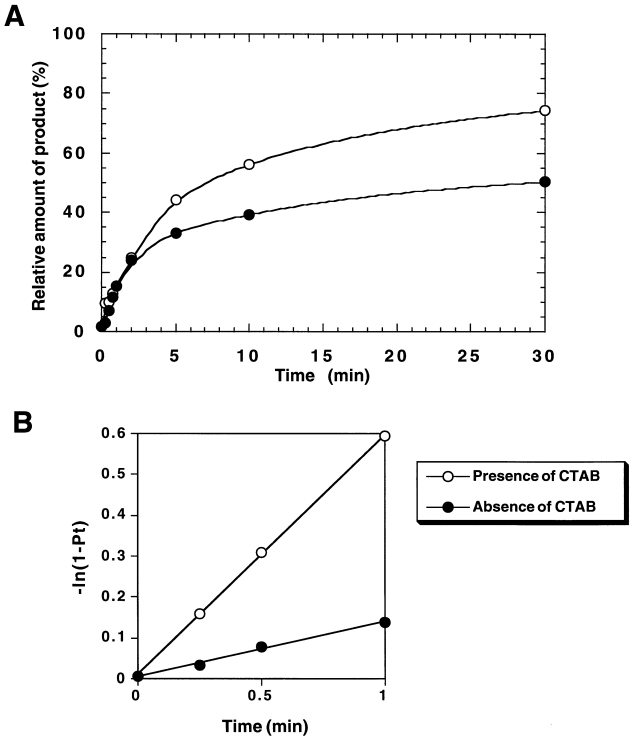

Fig. 5. Effects of CTAB on cleavage catalyzed by the 5 bp dimeric maxizyme. A: Time courses of cleavage reactions catalyzed by the 5 bp dimeric maxizyme in the presence (open circles) and in the absence (closed circles) of CTAB. B: The initial rates of the reactions catalyzed by the 5 bp dimeric maxizyme in the presence and in the absence of CTAB. Pt is the relative amount of product at any time [45].

activity of dimeric maxizymes in vitro [25]. Maxizymes with larger numbers of base pairs in the common stem II probably form a larger proportion of active dimers [25]. Our smallest maxizymes can form two G-C pairs in the common stem II and the dimeric maxizyme with the longest common stem II has five G-C pairs in this region. Since the 5 bp maxizyme has the highest activity [25] and since CTAB has been shown to enhance the activity of wild-type ribozymes by enhancing strand displacement [34–38], we first investigated the effect of CTAB on the 5 bp dimeric maxizyme targeted to GUC triplet-1 and GUC triplet-2 in HIV-1 tat mRNA (272 mer; Figs. 2 and 3B). Our preliminary results indicated that CTAB had a stimulatory rather than an inhibitory effect (without inhibition of dimerization) when the relatively long 272-meric HIV-1 tat mRNA was used as the substrate (Fig. 2A). In order to quantitate the stimulation by CTAB, we then used a short substrate of 19 nucleotides, S19, that corresponded to part of the HIV-1 tat mRNA. This substrate is cleaved into a 10-meric 5′-side product (labeled P in Fig. 4) and a 9-meric 3′-side product by the dimeric maxizyme.

We performed a kinetic study of reaction mixtures that contained 50 mM Tris-HCl (pH 8.0), 10 mM NaCl, and 25 mM $MgCl_2$, at 37°C under single-turnover conditions, using a fixed concentration of the 5 bp maxizyme that was close to its apparent K_m of 0.22 μM [25] and a fixed concentration of CTAB (50 μM) that was slightly above its critical micelle concentration (CMC). As shown in Fig. 5, CTAB not only increased the yield of products (Fig. 5A) but it also enhanced the initial rate (Fig. 5B). We recorded values of $k_{obs} = 0.13$ min^{-1} and $k_{obs} = 0.55$ min^{-1}, respectively, in the absence and in the presence of CTAB. These results suggested that CTAB enhanced the association step (k_{assoc} in Fig. 4) of the reaction since we followed the reactions under so-called k_{cat}/K_m conditions and since CTAB is known to inhibit the chemical cleavage step (k_{cleav}) [35,37]. We obtained analogous results with the long HIV-1 tat mRNA substrate (272-mer; data not shown).

3.2. Kinetic analysis of the reaction catalyzed by a 2 bp dimeric maxizyme targeted to GUC triplet-2 of HIV-1 mRNA

Since CTAB had a stimulatory effect on the reaction catalyzed by the 5 bp maxizyme, we next examined its effect on the 2 bp dimeric maxizyme, which had two G-C pairs in the common stem II (Fig. 3A). Since the 2 bp maxizyme was expected to be less stable than the 5 bp maxizyme, we feared initially that CTAB might have an inhibitory effect on the activity of the 2 bp maxizyme because of its strand displace-

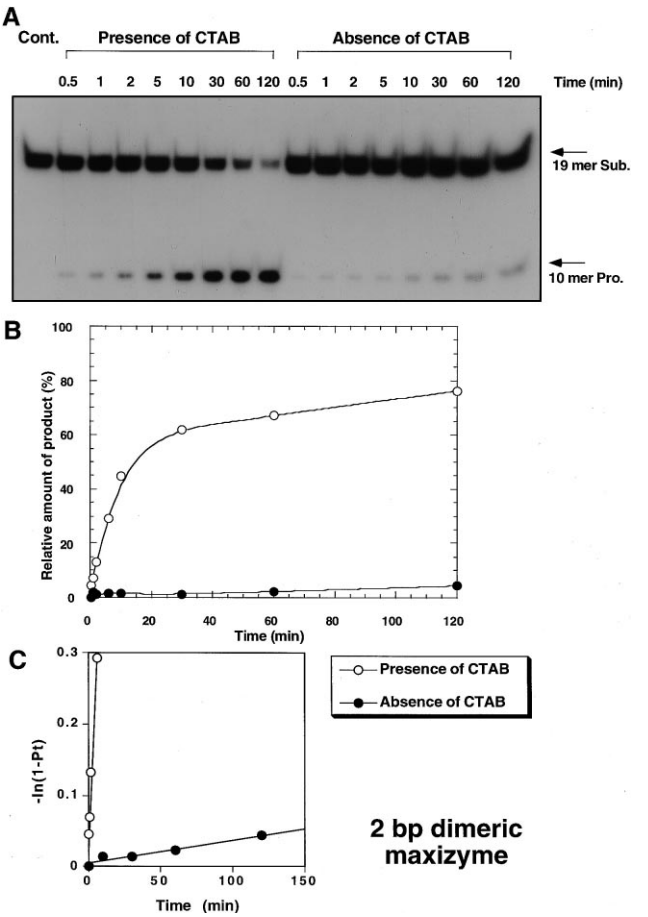

Fig. 6. Effects of CTAB on cleavage catalyzed by the 2 bp dimeric maxizyme. A: Autoradiogram showing the cleavage of the short substrate S19 by the 2 bp dimeric maxizyme in the presence (left) and in the absence (right) of 50 μM CTAB. B: Time courses of cleavage reactions catalyzed by the 2 bp dimeric maxizyme in the presence and in the absence of CTAB. C: The initial rates of the reactions catalyzed by the 2 bp dimeric maxizyme in the presence and in the absence of CTAB. Cont., control (labeled substrate only); Sub., substrate; Pro., product. For definition of Pt, see legend to Fig. 5.

ment activity [34–38]. Reactions were performed, as mentioned above, in 50 mM Tris-HCl (pH 8.0), 10 mM NaCl, and 25 mM $MgCl_2$ at 37°C, under single-turnover conditions and with a fixed concentration of the 2 bp maxizyme that was close to its apparent K_m of 1.0 μM [25]. We used a fixed concentration of CTAB (50 μM) and the same 19-mer substrate (S19) as described above. The results of the analysis are shown in Fig. 6. To our surprise, CTAB had a significant stimulatory effect (Fig. 6A). CTAB not only increased the yield of products (Fig. 6B) but it also enhanced the initial rate (Fig. 6C) to a significant extent. We recorded values of $k_{obs} = 0.001$ min^{-1} and $k_{obs} = 0.1$ min^{-1}, respectively, in the absence and in the presence of CTAB. Thus, the rate of the reaction was increased 100-fold.

In the case of 2 bp heterodimeric maxizymes, the dimers are expected to generate a mixture of inactive (MzR•MzR) dimers (consisting of two identical forms of maxizyme right), inactive (MzL•MzL) dimers (consisting of two identical forms of maxizyme left), and the desired active (MzR•MzL) dimers (consisting of maxizyme right and maxizyme left). It is partly because of this mixed population of dimers that the activity of 2 bp maxizymes is so low, in the absence of CTAB, as compared with that of 5 bp maxizymes (Fig. 7A,B). In the case of 5 bp heterodimeric maxizymes, the sequence of the common stem II was designed such that the equilibrium concentration of the active complexes with perfect base pairing in the common stem II would be much higher than that of inactive complexes with only partial base pairing (Fig. 7B). Moreover, the 2 bp maxizymes themselves seem to be folded in a complicated manner, since the second derivative curves of their UV absorption spectra have more transitions, each of which reflects the melting of intra- or intermolecular base pairing interactions (Fig. 7C–F). Similar results, indicating that the 2 bp maxizymes fold in a complicated manner, were obtained with mixtures of 2 bp heterodimeric maxizymes [25]. Note that the dimeric structure is stabilized not only by the formation of two G-C pairs at the common stem II but also, very probably, by additional interactions that include two reversed-Hoogsteen G-A base pairs between G_8-A_{13} and A_9-G_{12}, and a non-Watson-Crick A_{14}-U_7 base pair that consists of one hydrogen bond, as indicated by dotted lines in Fig. 1. The extended stem II is stacked on the non-Watson-

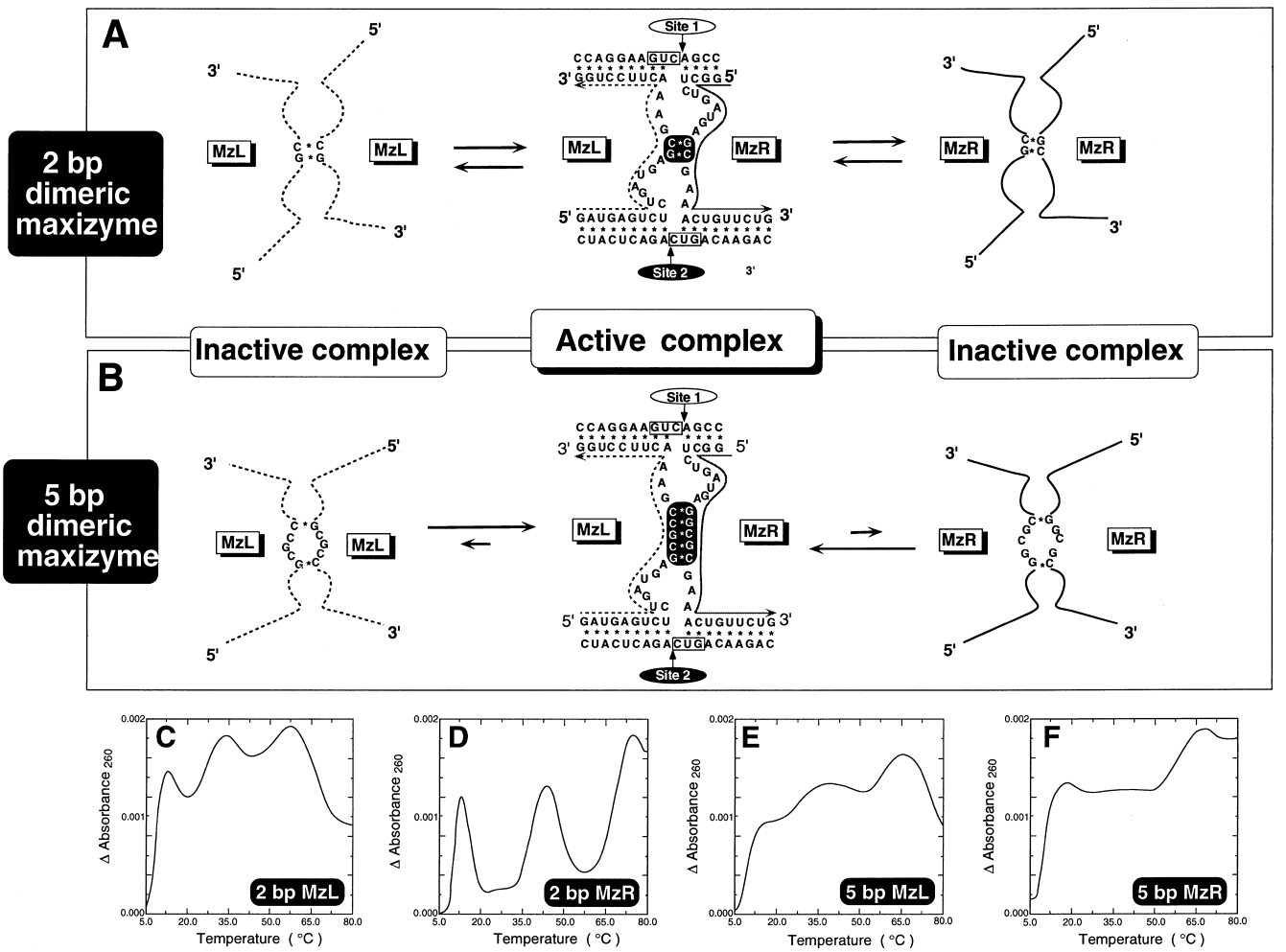

Fig. 7. Stimulation by CTAB of the conversion of inactive dimers to active dimers. A: Active and inactive dimeric forms of 2 bp dimeric maxizymes. A large fraction of the population of dimers is expected to be in an inactive form. B: The active and inactive dimeric forms of the 5 bp dimeric maxizymes. The formation of active forms is favored because perfect base pairing occurs only in the case of active complexes. C: Second derivative curve of the melting curve of the 2 bp maxizyme left (2 bp MzL). D: Second derivative curve for the 2 bp maxizyme right (2 bp MzR). E: Second derivative curve for the 5 bp maxizyme left (5 bp MzL). F: Second derivative curve for the 5 bp maxizyme right (5 bp MzR).

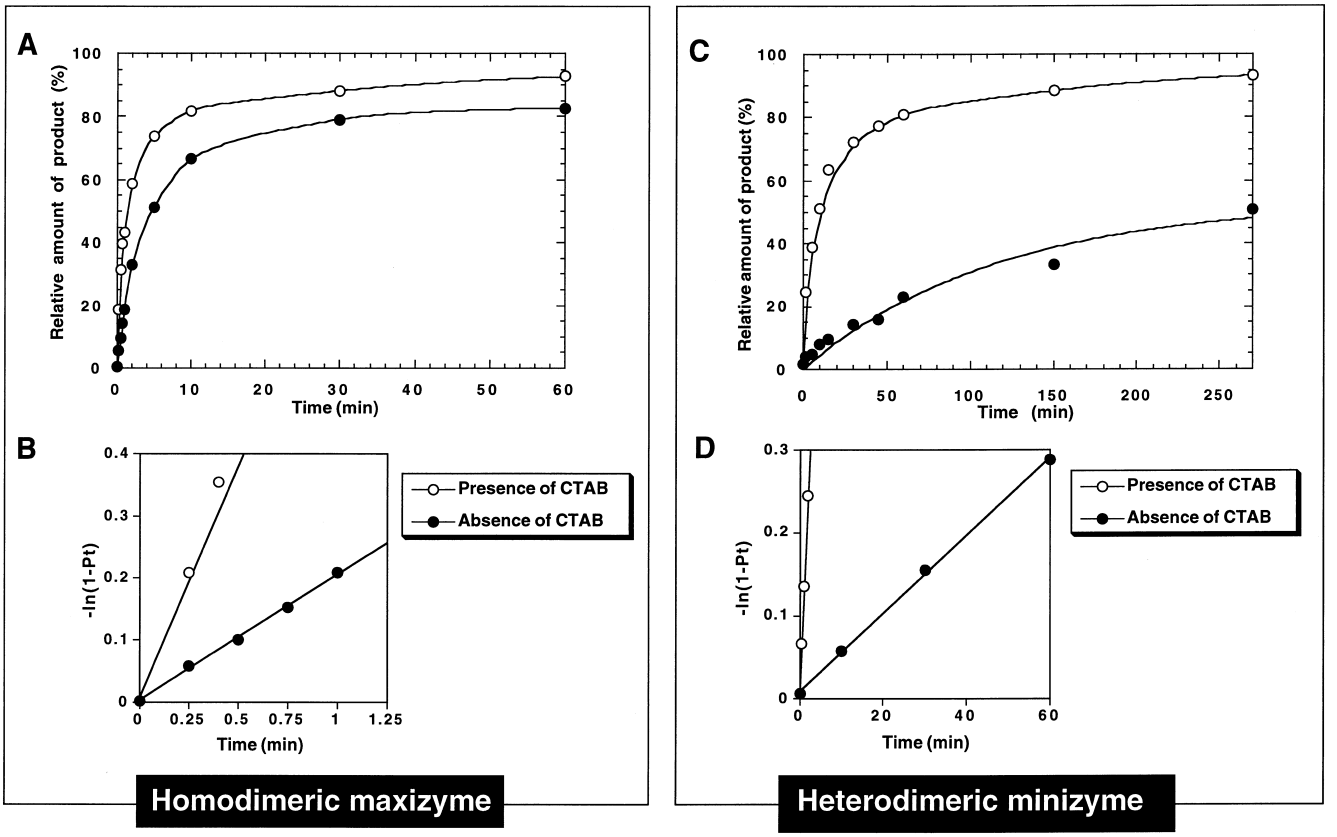

Fig. 8. Effects of CTAB on cleavage catalyzed by the homodimeric maxizyme and the heterodimeric maxizyme. A: Time courses of cleavage reactions catalyzed by the homodimeric maxizyme in the presence and in the absence of CTAB. B: The initial rates of reactions catalyzed by the homodimeric maxizyme in the presence and in the absence of CTAB. C: Time courses of cleavage reactions catalyzed by the heterodimeric maxizyme in the presence and in the absence of CTAB. D: The initial rates of the reactions catalyzed by the heterodimeric maxizyme in the presence and in the absence of CTAB. For definition of Pt, see legend to Fig. 5.

Crick base pair, $A_{15.1}$-$U_{16.1}$, with resultant formation of a pseudo-A-form helix by stems II and III [39–43].

CTAB apparently overcame the negative properties of 2 bp maxizymes. In the presence of CTAB, the activity of the 2 bp maxizyme approached that of the 5 bp maxizyme. The action of CTAB resembled that of an RNA chaperon [35], apparently inducing maxizymes that had misfolded (Fig. 7) to refold into an active conformation.

3.3. Comparison of the activities of homodimeric and heterodimeric maxizymes targeted to S11

CTAB facilitated the conversion of inactive dimers to active dimers (Fig. 7A,B). In order to examine whether the CTAB-mediated activation was due solely to the conversion of mispaired dimeric maxizymes to correctly paired dimeric maxizymes, we extended our study to include homodimeric maxizymes [4,43]. The homodimeric maxizyme we chose (homodimer; Fig. 1B) cleaved the 11-meric substrate (S11). The corresponding heterodimeric maxizyme (heterodimer; Fig. 1C) was also able to cleave the same S11 substrate, albeit at one site only. We did not expect the homodimeric maxizyme to be active in its monomeric configuration (Fig. 1B, left) since all active maxizymes seem able to form favorable base pairs at the common stem II.

We used the same reaction conditions as described above with fixed concentrations of maxizymes that were close to the apparent K_m values of 5.0 μM (Fig. 8A,B) and 0.5 μM (Fig. 8C,D), respectively [4], for the homodimer and heterodimer. In the case of the homodimer (Fig. 8A,B), we recorded values of $k_{obs} = 0.20$ min^{-1} and $k_{obs} = 0.75$ min^{-1}, respectively, in the absence and in the presence of CTAB. Even in the case of the homodimeric maxizyme, CTAB increased the rate up to 3-fold. This finding is in accord with the previous conclusion [34–38] that CTAB enhances the annealing of a ribozyme to its substrate. It is also possible that in this case, CTAB induced correct folding of intramolecularly misfolded maxizymes, as demonstrated in the case of 2 bp and 5 bp dimeric maxizymes (Fig. 7C–F).

With the heterodimeric maxizymes (Fig. 8C,D), the enhancement by CTAB was much greater (36-fold) and we recorded values of $k_{obs} = 0.0045$ min^{-1} and $k_{obs} = 0.16$ min^{-1}, respectively, in the absence and in the presence of CTAB. Even though the homodimer and the heterodimer had the same number of G-C pairs in the common stem II and even though they were directed at the same target, in other words, they were directed at the same substrate-binding site, it was apparent that there was difference in the extent of enhancement of their activities by CTAB. The greater enhancement by CTAB of activities of the heterodimeric maxizyme indicates that higher concentrations of inactive complexes, such as MzR•MzR and MzL•MzL (Fig. 4), were present in the absence of CTAB and were converted to active complexes (MzR•MzL) by CTAB.

We examined the effects of CTAB not only under k_{cat}/K_m

conditions with low concentrations of maxizymes but also under k_{cat} conditions. Even when concentrations of maxizymes were five-fold higher than their K_m values, we observed relatively small stimulatory effects in the presence of CTAB, on the cleavage mediated by both homodimeric and heterodimeric maxizymes (data not shown). In contrast, in a previous report Herschlag et al. [35] stated that CTAB inhibited the rate of the chemical cleavage step in a reaction catalyzed by a native hammerhead ribozyme. However, in the case of our dimeric maxizymes, even under the above-mentioned conditions significant concentrations of unfavorable intra- or inter-molecularly bonded structures must have been present, as they were under k_{cat}/K_m conditions. Therefore, it appears that added CTAB was able to refold inactive conformers to yield active conformation during the incubation for several minutes at 37°C that preceded the addition of an appropriate amounts of $MgCl_2$.

In summary, we demonstrated that CTAB enhanced the activity of both homodimeric and heterodimeric maxizymes, being more efficient in the latter case, under both k_{cat} and k_{cat}/K_m conditions. CTAB appeared to act via enrichment for active complexes of maxizymes under all conditions examined.

4. Conclusion

It was believed initially that minizymes are significantly less active than the corresponding full-sized ribozymes. However, the activities of our dimeric maxizymes are equal to those of parental hammerhead ribozymes, despite their smaller size [4,25]. Moreover, for cleavage of a long substrate, namely, the tat mRNA transcribed from HIV-1, synthetic maxizymes are more effective than full-sized ribozymes [25]. The present study demonstrates that the activities of dimeric maxizymes can be further stimulated by CTAB. The strand displacement activity of CTAB appears to enhance the conversion of inactive misfolded maxizymes, to active appropriately folded forms. In vivo, various facilitators exist that enhance strand displacement reactions similarly to CTAB, and they are expected to have stimulatory effects on the activities of dimeric maxizymes. Even maxizymes with a short stem II such as 2 bp dimeric maxizymes, which tend to form inactive structures in vitro, were found to have significant activity in the presence of CTAB, suggesting that they might be useful in vivo. In fact, in a separate experiment, in which we expressed 2 bp dimeric maxizymes in HeLa cells under the control of the pol III promoter, the maxizymes exhibited high-level activity that was at least as high as that of 5 bp dimeric maxizymes [26]. These observations further strengthen the conclusion reached in the present study that various kinds of facilitator in vivo that function similarly to CTAB might enhance the activity of dimeric maxizymes. Thus, dimeric maxizymes [4,25,26,43,44] should be considered as potentially powerful gene-inactivating agents in vivo. Moreover, CTAB appears to be useful in predicting activities of ribozymes in vivo.

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