Biochemistry (Moscow), Vol. 68, No. 11, 2003, pp. 1231-1238. Translated from Biokhimiya, Vol. 68, No. 11, 2003, pp. 1530-1539.

Properties of Antigenomic Hepatitis Delta Virus Ribozyme That Consists of Three RNA Oligomer Strands

T. I. Belianko, V. A. Alekseenkova, L. P. Savochkina*, M. A. Lukin, and R. Sh. Beabealashvilli

Russian Cardiology Research and Development Complex, Russian Academy of Medical Sciences, 3-ya Cherepkovskaya ul. 15a, Moscow 121552, Russia; fax: (7-095) 414-6699; E-mail: lasav@cardio.ru

Received October 8, 2002
Revision received November 18, 2002

Abstract—A three-strand ribozyme, a derivative of antigenomic hepatitis delta virus (HDV) ribozyme, which consists of subfragments of 16 (L), 17 (S), and 33 nucleotides (B), has been constructed. The ternary B–L–S complex formed by the subfragments in stoichiometric ratio was able to catalyze a self-cleavage reaction. Kinetics of this reaction exhibited biphasic behavior and the same parameters as in the case of natural *cis*-ribozyme. Study of kinetics of reaction initiated by adding various reaction components and the study of binary complex formation between subfragments B and L, B and S, and also ternary B–L–S complex formation revealed that: 1) in the presence of Mg^{2+}, B and S form a stoichiometric complex, L and S do not form complex at all, while B and L form 2 types of complexes, probably B–L and 2B–L; and addition of S subfragment prevented the formation of the latter complex; 2) the reaction initiated by S subfragment proceeds much slower than that initiated by other components pointing to the possibility that in the absence of S L may form a nonproductive complex with B, which is slowly displaced by S followed by productive ternary complex formation. Dissociation constants for binary B–L, B–S and ternary B–L–S complexes have been estimated.

Key words: ribozyme, HDV, subfragments, association, kinetic

HDV-ribozyme is a catalytic RNA, a constituent of the hepatitis D genome, functioning in mammalian cells. In the presence of bivalent metal ions it catalyzes the reaction of self-cleavage [1-3].

According to the accepted pseudoknot model [4], the HDV-ribozyme molecule comprises three functional subfragments: S fragment containing a cleavage site (substrate), substrate binding B subfragment, and L subfragment which presumably forms the active site of the ribozyme (together with the B subfragment). It was previously shown that the phosphodiester bond could be cleaved between either subfragment, which then associates together forming a bimolecular *trans*-ribozyme structure which can also catalyze self-cleavage as the initial *cis*-ribozyme [3, 5-8].

Use of physical methods for studies of structure of HDV-ribozyme and of self-cleavage reactions require a small ribozyme that consists of several subfragments. We have synthesized an analog of antigenomic HDV-ribozyme that consists of three RNA oligomers: substrate subfragment (S) of 17 nucleotides, and catalytic L and B subfragments of 16 and 33 nucleotides in length, respectively. Thus, the total length of this three-strand ribozyme was 66 nucleotides. For construction of this ribozyme we used the same principles of structure calculation that had been used before for construction of permutated variants of *cis*-ribozyme [9, 10].

The study of properties of the constructed *trans*-ribozyme revealed that like single-strand *cis*-ribozymes and double-strand *trans*-ribozymes described earlier, the three-strand ribozyme also exhibits biphasic reaction kinetics, the reaction rate depending on Mg^{2+}, formamide, pH, and temperature. However, some of the parameters studied markedly differed from those found in the literature, including those for a three-strand ribozyme [11].

MATERIALS AND METHODS

Chemicals. Ribooligonucleotides L, S, and B were synthesized by the standard protocol [12] using β-cyanoethyl phosphoramidites of corresponding nucleotides as monomers; their 2′-hydroxyl was substituted by *tert*-butyldimethylsilyl group. Synthesis was carried out using porous glass from Fluka (Germany) (pore size of 500 Å), carrying corresponding deoxynucleotides, and so the resultant oligoribonucleotides contained

* To whom correspondence should be addressed.

0006-2979/03/6811-1231$25.00 ©2003 MAIK "Nauka/Interperiodica"

deoxynucleotide residues at the 3′-end. After synthesis they were treated as described by Wincott et al. [13], purified by electrophoresis under denaturing conditions in 15% polyacrylamide gel in the presence of 7 M urea, eluted from the gel, and desalinated by chromatography on a NenSorb 20 column (NEN Co., USA). The nucleotide sequence of synthesized oligomers was confirmed by ribonucleotide mapping as described earlier [14] using T4-polynucleotide kinase (Biosan, Russia) and [γ-^{32}P]ATP with specific activity 5000 Ci/mmol (State Research Center of the Russian Federation FEI, Obninsk, Russia). ^{32}P-Labeled oligonucleotides were also purified by electrophoresis under denaturing conditions in 15% polyacrylamide gel in the presence of 7 M urea, eluted, and desalinated by chromatography on the NenSorb 20 column. Oligonucleotide concentration was determined spectrophotometrically at 260 nm.

Electrophoresis was carried out at 50°C in 10-15% polyacrylamide gel in the presence of 7 M urea under denaturing conditions. The electrophoretic system contained 90 mM Tris-borate buffer, pH 8.5, and 25 mM EDTA. The association/dissociation of ribozyme subunits was studied by means of electrophoresis under nondenaturing conditions in 20% polyacrylamide gel containing 50 mM Tris-acetate buffer, pH 7.5, and 11 mM MgAc. The electrophoretic procedure was carried out at 20°C using a thermostatic 2050 MIDGET Electrophoresis Unit (LKB, Sweden).

The cleaving activity was analyzed in 50 mM Hepes-NaOH buffer, pH 7.5. The mixture of ^{32}P-labeled substrate RNA and RNA oligomers constituting enzyme was incubated at 50°C for 10 min. The reaction was initiated by adding preheated $MgCl_2$ (final concentration 10 mM) or a subfragment required for complete mixture. Precise incubation time is indicated at legends to corresponding figures. Effect of pH on the reaction rate was studied using 50 mM formic acid, 50 mM sodium acetate, 50 mM Tris-acetate, 50 mM carbonate/bicarbonate buffer; pH values of the buffers being determined at 50°C. The effect of formamide on catalytic activity of the three-strand ribozyme was studied after its preincubation with ribozyme for 10 min in the reaction mixture. The reaction initiated by adding $MgCl_2$ (final concentration 10 mM) was then terminated by a stop solution containing 85% formamide, 50 mM EDTA, 0.01% bromophenol, and 0.01% xylene cyanole. The reaction products were analyzed by electrophoresis in 18% polyacrylamide gel in the presence of 7 M urea followed by subsequent radioautography using PhosphorImager SI (Molecular Dynamics, USA) and the software ImageQuant 5.1. All experiments were repeated at least three times, and experimental error did not exceed 20%.

Kinetic curves were analyzed using the system of electronic tables in Microsoft Excel. Experimental curves were approximated to polynomial of the fourth degree ($R^2 > 0.95$) and initial reaction rate (v_0) was determined as the coefficient at the linear polynomial member.

RESULTS AND DISCUSSION

Ribozyme structure. The three component ribozyme constructed in the present study differed from natural single strand antigenomic HDV-ribozyme by the nucleotide sequence due to deletions in *J1/2* and *s4* sites and nucleotide substitutions in the substrate containing S subfragment (Fig. 1). The catalytic part of this ribozyme includes two subfragments, B and L, both subfragments carrying additional nucleotides on their termini, and the double strand *s2* site presumably formed during their interaction is longer than the natural one by a G–C pair.

Dependence of substrate cleavage rate on concentration of subfragments B, L, and S. We have investigated the dependence of substrate cleavage rate on concentration of ribozyme components using their equal content and also excess of catalytic part L + B over the substrate part (S) under conditions of their simultaneous addition.

Figure 2a shows the kinetics of substrate cleavage at 50°C and various concentrations of ribozyme subfragments, and Fig. 2b shows the dependence of v_0 on ribozyme concentrations. Presentation of the dependence of v_0 versus substrate concentration in double reciprocal plots (Fig. 2b, insert) gave V_{max} value of 6.2 ± 1% of cleaved substrate per 1 min and K_m value of 0.162 ± 0.01 μM subfragments, respectively (at 50°C). Similar V_{max} and K_m values for S were obtained with excess of L and B (Fig. 2c). The K_m values obtained in the present study are close to those reported for bimolecular *trans*-ribozyme (0.5-0.7 μM) [7, 15].

The results of kinetic studies revealed a region of independence of cleavage reaction rate on ribozyme subfragment concentration. This is the region of concentrations exceeding 1 μM. So all subsequent experiments on evaluation of the effects of various factors on the reaction of ribozyme cleavage were carried out at the concentration of subfragments of 1.25 μM.

Dependence of ribozyme cleavage reaction rate on Mg^{2+} concentration. Figure 3a shows that at any concentration of Mg^{2+} there was biphasic kinetic curve and an increase of Mg^{2+} concentration (within the concentration range used) caused an increase in initial rate of the cleavage reaction. Figure 3b shows the dependence of v_0 for the three-strand ribozyme on Mg^{2+} concentration. There was clear dependence of v_0 on Mg^{2+} in the range of ion concentration up to 30 mM. Calculation of K_m and V_{max} values using data of Fig. 3 presented in double reciprocal plots (Fig. 3b, insert) gave the following results: V_{max} = 7.3 ± 1%/min, K_m for Mg^{2+} = 10.5 mM.

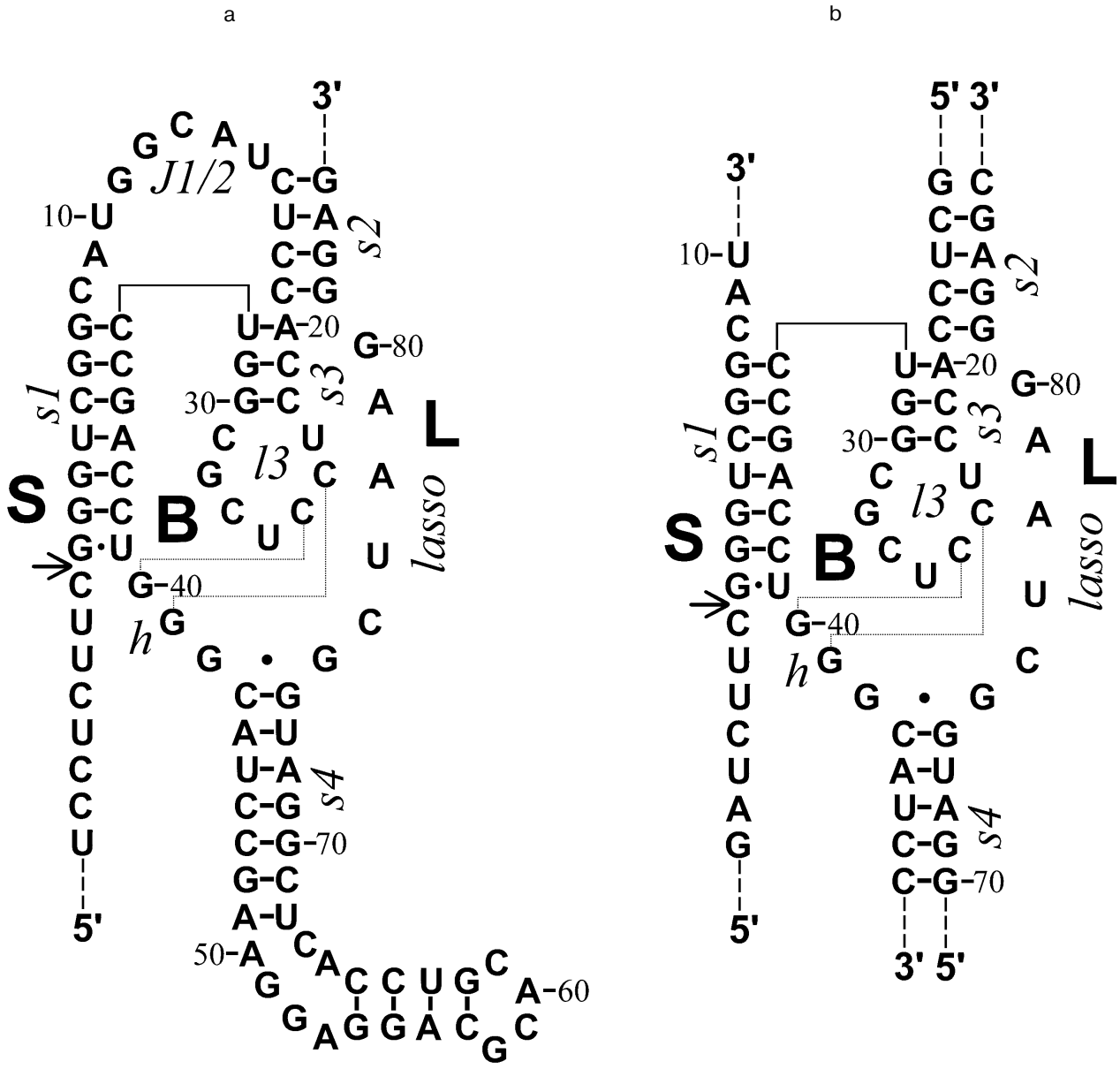

Fig. 1. Nucleotide sequence and putative secondary structure of natural HDV-ribozyme (a) and its three-component variant L–S–B used in this study (b). Secondary structure and nucleotide numeration correspond to those from Perrotta and Been [4]. Arrows indicate the position of phosphodiester bond cleavage. B and L are sequences forming the catalytic site of the ribozyme, whereas S is the ribozyme subfragment containing substrate; *s1*, *s2*, *s3*, and *s4* are double-strand sites of the ribozyme molecule; *J1/2*, *J1/4* (*hinge*, *h*), *J4/2* (*lasso*) are single-strand sequences joining *s1* and *s2*, *s1* and *s4*, *s4* and *s2*; *l3* is a single-strand sequence linking 5′- and 3′-ends of *s3*.

Similar dependence of the cleavage rate on Mg^{2+} concentration was found for all families of *cis*-ribozymes (SBL, LBS, and BLS) irrespective of their topology [10]. Similar results were also obtained for bimolecular *trans*-ribozyme [16]. However, studies of the other HDV-like three-component *trans*-ribozyme by Sakamoto et al. [11] revealed different Mg^{2+}-sensitivity of the reaction.

The pH dependence of ribozyme cleavage. The ribozyme cleavage activity was studied in the pH range of 3-10. Samples were incubated at 50°C for 5 min in the presence of 10 mM $MgCl_2$. Figure 4 shows that the cleavage was maximal at pH 5.5-6.0, and at pH 6.0-6.75 it slightly decreased and remained at a plateau up to pH 7.75. At pH below 5.5 and higher than 8.0 the cleavage significantly reduced. At pH below 4.0 and higher than 9.0 the ribozyme was completely inactive.

Similar pH dependence of the three-component ribozyme self-cleavage reaction differs from previously

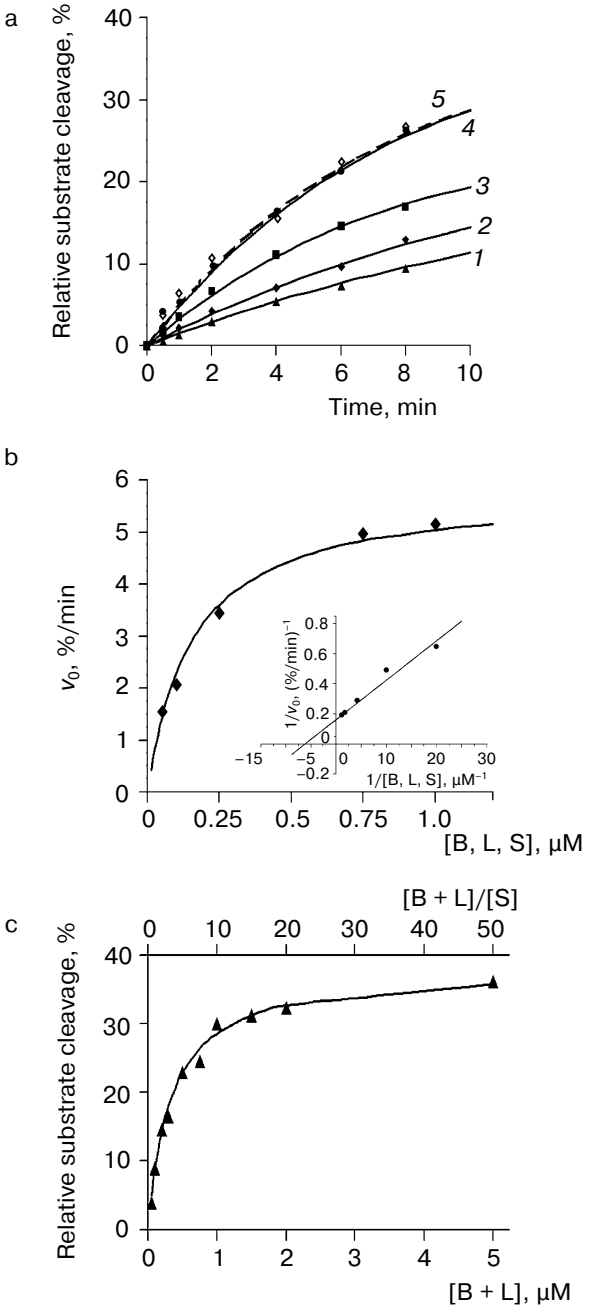

Fig. 2. Dependence of the cleavage reaction rate on ribozyme concentration. a) Kinetics of ^{32}P-labeled substrate S cleavage during mixing of subfragments (L + B) and S at the ratio 1 : 1 and concentrations 0.05 (*1*), 0.1 (*2*), 0.25 (*3*), 0.75 (*4*), and 1 μM (*5*). The abscissa is incubation time, ordinate is proportion of substrate molecules with cleaved phosphodiester bond (%). b) Dependence of initial reaction rate on ribozyme concentration. The ordinate is initial reaction rate v_0, expressed as % of cleaved substrate per 1 min (%/min). The insert shows data of Fig. 2b presented in a double reciprocal plot. c) Dependence of substrate S cleavage on concentration of (B + L) subfragments at constant substrate concentration of 0.1 μM and (B + L) concentrations varied within the range 0.05 to 5.0 μM. Abscissa is the ratio (B + L)/S (top) and concentration of B + L (bottom). The ordinate is relative substrate cleavage (%).

reported data for other HDV-like ribozymes [17], including a three-strand ribozyme [11]. However, the pH behavior of the ribozyme found in this study is similar to that of natural HDV-ribozyme [18] and its analogs [10]. Some double-strand ribozymes exhibit such pH dependence [16, 19]. We have also found similar pH dependence for a double-strand *trans*-ribozyme (paper in preparation).

Effect of formamide on the self-cleavage. Denaturing agents can cause increasing or decreasing of catalytic activity of *cis*-ribozymes, HVD-ribozyme derivatives [20-23]. As expected, formamide did not stimulate self-cleavage of double-strand *trans*-ribozymes [24]. Figure 5 shows that low concentrations of formamide (<1 M) caused a sharp decrease in the cleavage activity. In contrast to *cis*-ribozyme analogs studied earlier [10], this effect insignificantly differed after 5 and 45 min of incubation. It is possible that even low formamide concentration prevented association of ribozyme subfragments by influencing their conformation. However, results of studies of two-component ribozyme suggest that low concentrations of formamide may promote an association process (paper in preparation).

Temperature dependence of ribozyme self-cleavage. Certain evidence exists that HDV-ribozyme may be catalytically active not only at 37°C, but also at higher temperatures of the incubation medium [17, 25-27]. In some cases an increase in temperature by 10°C is accompanied by 10-15-fold increase in the rate constant of the self-cleavage reaction [28].

For determination of temperature optimum for catalytic activity, the three-part ribozyme self-cleavage reaction was investigated in the temperature range from 28 to 60°C. Figure 6 shows that the increase in temperature from 30 to 50°C caused increase in the reaction rate, reaching maximum value at 50°C. Subsequent increase in temperature of the incubation medium resulted in a decrease in cleavage rate. Figure 6b shows the temperature dependence of reaction rate constant.

Dependence of ribozyme cleavage activity on order of addition of subfragments to the reaction mixture. Studying conditions required for the three-part ribozyme formation, we found that the order of addition of the ribozyme subfragments into the reaction mixture is essential for manifestation of catalytic activity. The reaction initiated by addition of Mg^{2+} or L subfragment was characterized by higher reaction rate than that initiated by subfragment S addition.

Figure 7 shows the dependence of reaction rate on order of addition of ribozyme components. Irrespective of concentration of the interacting subfragments, the initial cleavage rate was 3.5 times higher when the reaction was initiated by addition of Mg^{2+} or L subfragment.

It is possible that the interaction of B and S subfragments in the presence of Mg^{2+} causes formation of B–S complex with "correct" subfragment folding, so that added L subfragment is immediately positioned at a site

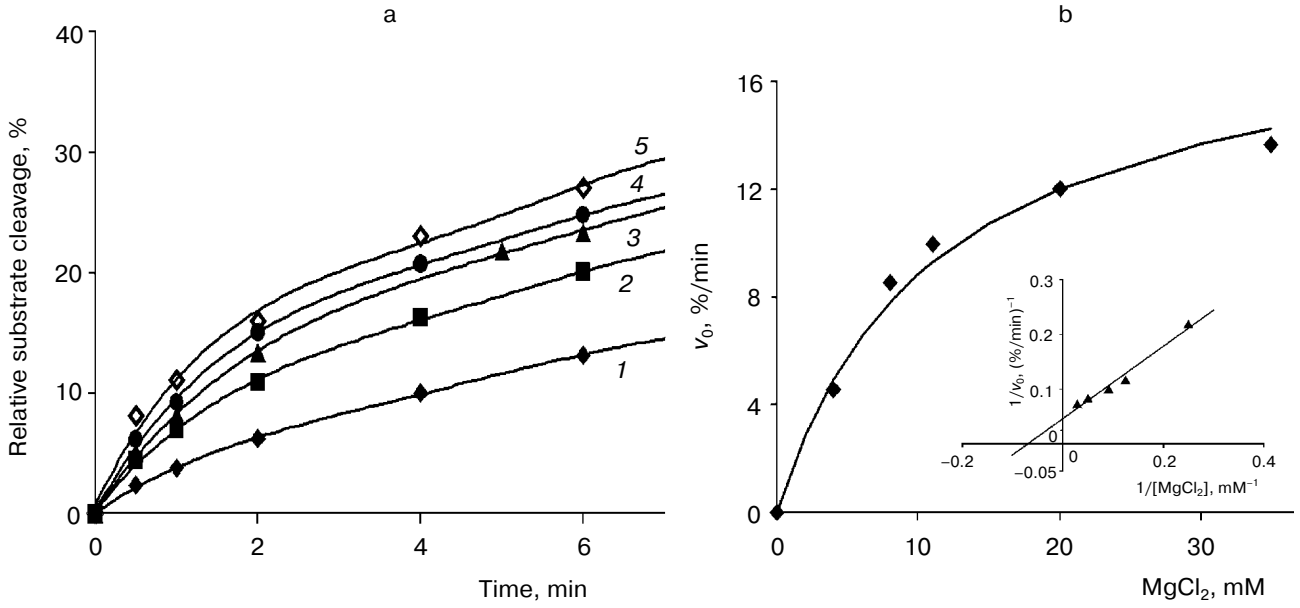

Fig. 3. Dependence of relative cleavage of ^{32}P-labeled substrate S on $MgCl_2$ concentration. The mixture of three 1.25 μM subfragments was incubated at 50°C in the cleavage buffer; the reaction was initiated by adding $MgCl_2$. a) Kinetics of substrate cleavage at different concentrations of $MgCl_2$: 4 (*1*), 8 (*2*), 10 (*3*), 20 (*4*), and 35 mM (*5*). The ordinate is relative substrate cleavage (%). b) Dependence of v_0 on $MgCl_2$; the insert shows presentation of data of Fig. 3b in a double reciprocal plot.

required for active complex formation. Addition of Mg^{2+} to the mixture of all three subfragments gave the same result as preincubation of B and S subfragments.

Interaction of B and L subfragments could yield L–B complex with "incorrect" conformation and formation of productive complex requires its rearrangement, which occurs after addition of S subfragment. This is accompanied by reduced reaction rate.

These results of the dependence of productive complex formation on order of addition of the subfragments into the reaction medium were further validated by using PAGE under non-denaturing conditions.

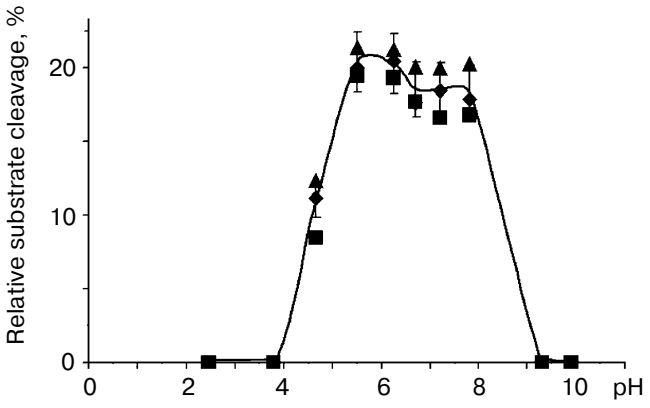

Fig. 4. The pH dependence of substrate cleavage. The mixture of 1.25 μM ^{32}P-labeled substrate S and subfragments L and B was incubated at 50°C for 5 min in different buffers within the range of pH 2.0-10.0. The composition of buffers was described in the "Materials and Methods" section. The ordinate shows relative substrate cleavage (%).

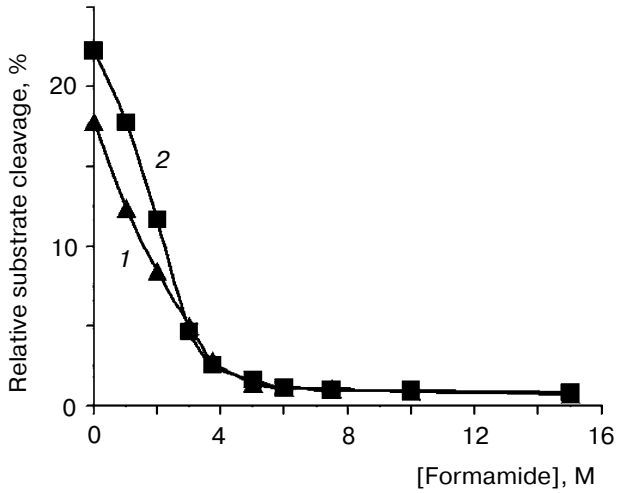

Fig. 5. Effect of formamide on substrate cleavage. The mixture of 1.25 μM ^{32}P-labeled substrate S and subfragments L and B was incubated at 50°C for 5 (*1*) and 45 min (*2*) in the presence of various concentrations of formamide. The cleavage reaction was initiated by adding $MgCl_2$ (final concentration 10 mM). The ordinate shows relative substrate cleavage (%).

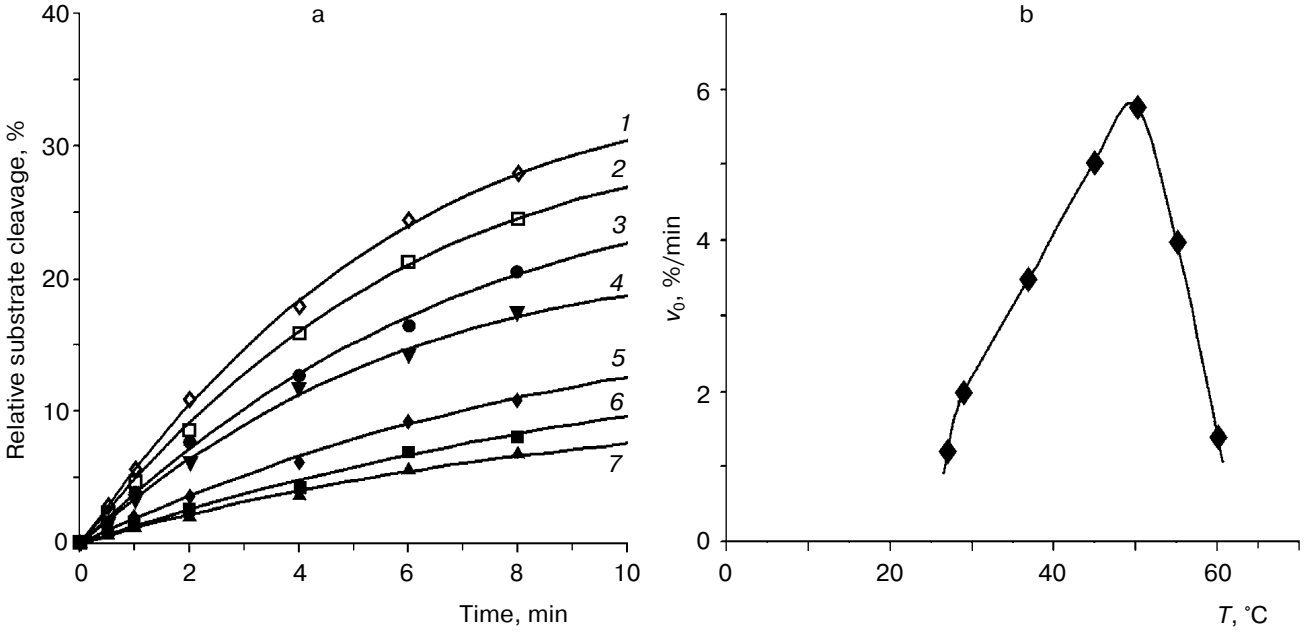

Fig. 6. Temperature dependence of ^{32}P-labeled substrate S cleavage. The mixture of 1.25 μM ^{32}P-labeled substrate S and subfragments L and B was incubated at various temperatures. a) Kinetics of substrate S cleavage at: 50 (*1*), 45 (*2*), 55 (*3*), 37 (*4*), 29 (*5*), 60 (*6*), 27°C (*7*). b) Dependence of initial rate of cleavage (v_0) on temperature of the incubation medium, based on results in Fig. 6a.

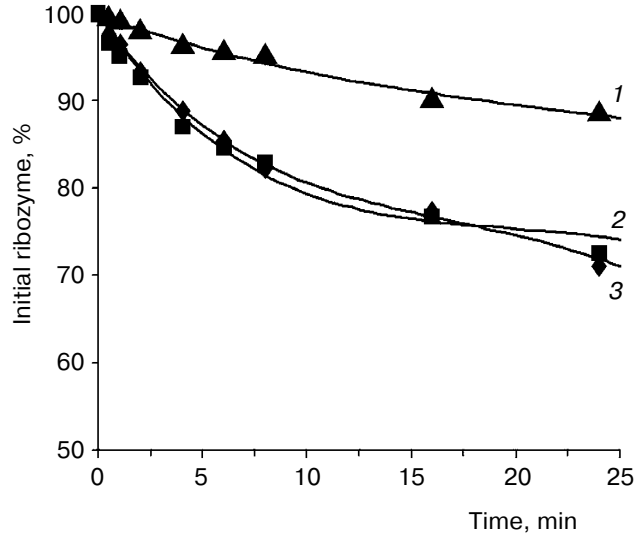

Fig. 7. Dependence of cleavage yield of the ribozyme on order of subfragment addition to the reaction medium. Concentration of the subfragments was 1.25 μM. *1*) B and L subfragments were preincubated at 50°C in the cleavage buffer in the presence of 10 mM $MgCl_2$; the reaction was initiated by adding S subfragment. *2*) Preincubation of B and S subfragments was carried out under the same conditions, but the reaction was initiated by adding L subfragment. *3*) All ribozyme subfragments (S, B, L) were preincubated at 50°C in the cleavage buffer, the reaction was initiated by adding $MgCl_2$ (to final concentration 10 mM).

Study of the interaction between ribozyme subfragments by means of electrophoresis under non-denaturing conditions. In these experiments we used low concentrations of subfragments. The ^{32}P-labeled subfragment B concentration was constant (44 nM), whereas concentrations of other subfragments were varied from 4 to 88 nM. Resulting products were separated by electrophoresis under non-denaturing conditions in the presence of Mg^{2+}. Analysis of electrophoregrams revealed the dependence of complex formation on subfragment concentration and calculation of dissociation constants for these complexes.

Preliminary data revealed that in the presence of Mg^{2+} subfragment B may form complexes with both with L and S subfragments (Fig. 8). Within the incubation period studied (5-200 min) the complex formation did not depend on the incubation time of the mixture, but did depend on the ratio between components. It should be noted that in the absence of Mg^{2+} in the reaction mixture and in electrophoresis buffer, complex formation from the subfragments was registered only at 4°C. At 50°C even the presence of Mg^{2+} does not promote maintenance of L–B complex, whereas S and B subfragments form a stable complex. Similar dependence was also found for the double-strand *trans*-ribozyme (paper in preparation). All subsequent experiments were carried out at 37°C to reduce the degree of substrate cleavage and to detect stable complexes.

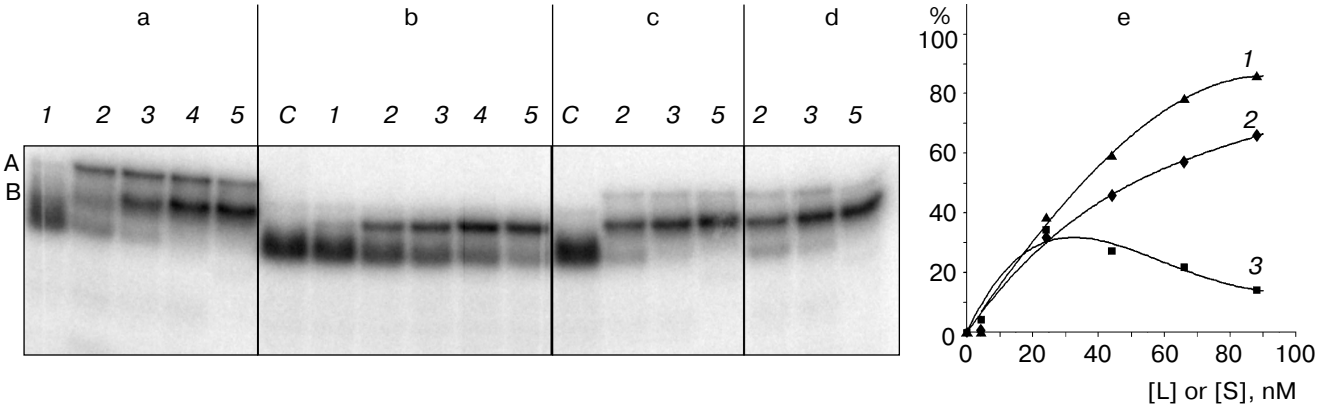

Fig. 8. Study of interaction of separate subfragments of the ribozyme. ^{32}P-Labeled subfragment B (44 nM) was incubated with subfragments L (a), S (b), or L and S (c, d) used in concentrations: 4.4 (*1*), 22 (*2*), 44 (*3*), 66 (*4*), and 88 nM (*5*). The incubation was carried out at 37°C for 90 min (a, b); c) subfragment B was incubated with subfragment S (44 nM) for 30 min, and after subfragment L addition the mixture was incubated for 60 min; d) the mixture of B and S (44 nM each) and L (as indicated) was incubated for 90 min. All incubation mixtures contained 50 mM Hepes and 10 mM $MgCl_2$; e) graphic presentation of results presented in Fig. 8 (a and b): the dependence of subfragment B amount in the complex (% of initial amount) formed during interaction between B and L subfragments (B–L complex) (*1*), B and S subfragments (B–S complex) (*2*), or B–L–B complex (*3*) on initial concentration of L or S subfragments. C, control.

Interaction of B and L subfragments. Incubation of B and L yielded two types of complexes of lower (A) and higher (B) mobility; their relative content depended on concentrations of components (Fig. 8a). Increase in L subfragment concentration resulted in transition of one type of complex into the other. For example, at L/B ratio 1 : 2 (22 and 44 nM, respectively) there was predominant formation of complex type A, whereas at the ratio 1 : 1 (concentration of each subfragment was 44 nM) complex B of higher electrophoretic mobility appeared. At L/B ratio 2 : 1 the content of B subfragment in complex B increased up to 85%. The calculated dissociation constant for complex B was 18 ± 5 nM.

Different mobility of the complexes formed suggests that excess of subfragment B favors formation of the complex type 2B–L, whereas excess of subfragment L favors formation of the complex type B–L. This suggestion is supported by results of an experiment in which (in contrast to Fig. 8a data) ^{32}P-labeled subfragment L was used as a marker of complex formation (due to identical pattern, these results are not shown).

Interaction of B and S subfragments yielded another pattern (Fig. 8b). In this case only one type of complex was formed with stoichiometry 1 : 1. The calculated dissociation constant was 50 ± 5 nM. Even 2-fold excess of S subfragment did not promote involvement of all subfragment B into complex formation. However, addition of subfragment L after the preincubation of the equimolar concentration of B and S subfragments (44 nM each) during 30 min resulted in total incorporation of subfragment B into the ternary complex at the component ratio 1 : 1 : 1 (Fig. 8c). The constant for subfragment L dissociation from the ternary complex was 8 ± 2 nM. The experiment employing ^{32}P-labeled subfragment S also revealed that B and S subfragments form associates under either conditions tested and at various concentrations of components (within the concentration range from 20 to 1000 nM), whereas subfragments S and L do not form complexes under any experimental conditions.

Interaction of all three components simultaneously added into the mixture did not cause total involvement of all components into the complex formation. At equimolar concentration of B, L, and S about 10% of subfragment B remained complex-free (Fig. 8d). This may be attributed to low concentration of components used. In fact, 2-fold excess of L subfragment added simultaneously with other subfragments and $MgCl_2$ resulted in binding of the total amount of B subfragment into the complex. Figure 8e shows the dependence of complex formation on concentration of subfragments.

These results confirmed the earlier observation that: 1) some ribozyme subfragments form double complexes characterized by lower affinity (or lower association constant) than in the case the full three-part ribozyme; 2) preincubation of B and S subfragments (prior to subfragment L addition) in the presence of Mg^{2+} favors quantitative association of all three subfragments.

The HDV-like ribozyme composed of three separate oligonucleotide subfragments may catalyze phosphodiester bond cleavage at the substrate part as the single-strand natural ribozyme. Reaction parameters are similar

for both ribozymes: kinetic curves of the cleavage reaction are characterized by biphasic behavior, and both ribozymes share the same dependence on Mg^{2+} and pH. However, as expected there are some differences, which may be attributed to necessity of association of subfragments of the three-strand ribozyme into one complex required for the cleavage reaction. Evidently, formamide addition to the reaction mixture would reduce cleavage reaction rate. In fact, even 1 M formamide markedly reduced the rate of the reaction.

Increase in temperature of the reaction medium above a certain limit would also prevented ribozyme subunit complex formation. This limit is the optimal temperature required for maximal cleavage; the three-strand ribozyme temperature optimum is at 50°C. Subsequent increase in temperature resulted in a sharp decrease of the reaction rate. In all our HDV-like permutated variants of the *cis*-ribozyme the cleavage rate also increased with increase in temperature and reached maximal values at 50°C. However, in one of the *cis*-ribozyme family (BLS) increase in temperature above 50°C caused further increase in catalytic activity, and the temperature optimum was observed at 60°C [10].

A remarkable property of the three-strand ribozyme investigated in the present study consists in rather strict dependence of its catalytic activity on order of ribozyme component addition. This phenomenon can be attributed to paired association of B and S subfragments which form complexes with the conformation required for "correct" folding of subfragment L.

The results of the present study suggest that under certain experimental conditions it is possible to obtain physically homogenous equimolecular associate of three ribozyme components that is indistinguishable from its monomolecular analog.

REFERENCES

1. Kuo, M. Y. P., Sharmeen, L., Dinter-Gottlieb, G., and Tailor, J. (1988) *J. Virol.*, **62**, 4439-4444.
2. Sharmen, L., Kuo, M. Y. P., Dinter-Gottlieb, G., and Tailor, J. (1988) *J. Virol.*, **62**, 2674-2679.
3. Been, M. D. (1994) *Trends Biochem. Sci.*, **19**, 251-256.
4. Perrotta, A. T., and Been, M. D. (1991) *Nature*, **350**, 434-436.
5. Perrotta, A. T., and Been, M. D. (1993) *Nucleic Acids Res.*, **21**, 3959-3965.
6. Branch, A. D., and Robertson, H. D. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 10163-10167.
7. Perrotta, A. T., and Been, M. D. (1992) *Biochemistry*, **31**, 16-21.
8. Kawakami, J., Yuda, K., Suh, Y. A., Kumar, P. K. R., Nishikawa, F., Maeda, H., Taira, K., Ohtsuka, E., and Nishikawa, S. (1996) *FEBS Lett.*, **394**, 132-136.
9. Alekseenkova, V. A., Savochkina, L. P., Timofeeva, A. V., Scrypina, N. A., Beabealashvilli, R. Sh., and Hoyman, H. (1999) *Mol. Biol. (Moscow)*, **33**, 595-597.
10. Alekseenkova, V. A., Nelianko, T. I., Timofeeva, A. V., Savochkina, L. P., Hoyman, H., and Beabealashvilli, R. Sh. (2001) *Mol. Biol. (Moscow)*, **35**, 879-899.
11. Sakamoto, T., Tanaka, Y., Kuwabara, T., Kim, M. H., Kurihara, Y., Katahira, Y., Katahira, M., and Uesugi, S. (1997) *J. Biochem.*, **121**, 1123-1128.
12. Damha, M. J., and Ogilvie, K. K. (1993) *Meth. Mol. Biol.*, **20**, 81.
13. Wincott, F., DiRenzo, A., Shaffer, C., Grimm, S., Tratz, D., Workman, C., Sweedeler, D., Gonzalez, C., Scaringe, S., and Usman, N. (1995) *Nucleic Acids Res.*, **23**, 2677-2684.
14. Maniatis, T. A., Fritsch, E. F., and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Lab., Cold Spring Harbor, N. Y.
15. Been, M. D., Perrotta, A. T., and Rosenstein, S. P. (1992) *Biochemistry*, **31**, 11843-11852.
16. Fauzi, H., Kawakami, J., Nishikawa, F., and Nishikawa, S. (1997) *Nucleic Acids Res.*, **25**, 3124-3130.
17. Wu, H.-N., and Lai, M. M. C. (1990) *Mol. Cell. Biol.*, **10**, 5575-5578.
18. Wu, H.-N., Lin, Y.-J., Lin, F.-P., Makino, Sh., Chang, M.-F., and Lai, M. M. C. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 1831-1835.
19. Shih, I.-H., and Been, M. D. (1999) *RNA*, **5**, 1140-1148.
20. Perrotta, A. T., and Been, M. D. (1998) *J. Mol. Biol.*, **279**, 361-373.
21. Perrotta, A. T., and Been, M. D. (1996) *Nucleic Acids Res.*, **24**, 1314-1321.
22. Wadkins, T., Perrotta, A. T., Ferre-D'Amare, A. R., Doudna, J., and Been, M. D. (1999) *RNA*, **5**, 720-727.
23. Gottlieb, P. A., Prasad, Y., Smith, J. B., Williams, A. P., and Dinter-Gottlieb, G. (1994) *Biochemistry*, **33**, 2802-2808.
24. Lee, C. B., Lai, Y. C., Ping, Y. H., Huang, Z. S., Lin, J. Y., and Wu, H. N. (1996) *Biochemistry*, **35**, 12303-12312.
25. Belinsky, M. G., and Dinter- Gottlieb, G. (1991) *Nucleic Acids Res.*, **19**, 559-564.
26. Sharmeen, L., Kuo, M. Y.-P., Dinter-Gottlieb, G., and Taylor, J. (1988) *J. Virol.*, **62**, 2674-2679.
27. Thill, G., Vasseur, M., and Tanner, N. K. (1993) *Biochemistry*, **32**, 4254-4262.
28. Rosenstein, S. P., and Been, M. D. (1990) *Biochemistry*, **29**, 8011-8016.