

# Ligation activity of fragmented ribozymes in frozen solution: implications for the RNA world

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## ABSTRACT

**A vexing difficulty of the RNA world hypothesis is how RNA molecules of significant complexity could ever have evolved given their susceptibility to degradation. One way degradation might have been reduced is through low temperature. Here we report that truncated and fragmented derivatives of the hairpin ribozyme can catalyze ligation of a wide variety of RNA molecules to a given sequence in frozen solution despite having little or no activity under standard solution conditions. These results suggest that complex RNAs could have evolved in freezing environments on the early earth and perhaps elsewhere.**

## INTRODUCTION

The RNA World (1–3) refers to an evolutionary period prior to coded peptide synthesis in which RNA may have been the major genetic and catalytic agent. If the prebiotic conditions were ‘warm and wet’ as is usually assumed, the RNA world hypothesis has a major difficulty. Under those conditions the RNA backbone undergoes rapid cleavage through transesterification. This reaction is accelerated by divalent metal ions, which would have been abundant in aqueous solutions (4,5). Thus, the evolution of RNA sequence complexity must have occurred under conditions in which RNA synthesis was more efficient than random degradation. Since the first ribozymes are likely to have been inefficient, the most straightforward way for RNA synthesis to outpace random degradation would be if conditions were such that degradation was slow. Degradation could have been reduced at low concentrations of divalent metal ions, low temperature, reduced water activity and upon adsorption to surfaces so as to stabilize phosphodiester bonds (4–9). Most of these conditions, and particularly low temperature, may have been available on the early Earth. Indeed, some investigators have argued that at the time of prebiotic evolution much of the

water in the oceans was frozen but underwent periodic melting due to large meteor impacts or volcanic activity (8–10).

Several potential prebiotic reactions are known to proceed in frozen solutions, including pyrimidine and purine synthesis from ammonium cyanide (11), formation of dinucleotides from adenosine 2', 3'-cyclic phosphate (12), and synthesis of polynucleotides from phosphor-imidazole-activated mononucleotides (13,14). Earlier, we observed that in frozen solutions, the hairpin ribozyme (HPR), a small catalytic RNA derived from the minus strand of the satellite RNA of tobacco ringspot virus, promotes Mg<sup>2+</sup>-independent ligation in *cis* (intramolecular ligation), while the cleavage reaction is inhibited (15). In the standard solution system, the HPR can either cleave substrates to generate RNA fragments with 5'-hydroxyl and 2', 3'-cyclic phosphate termini or ligate them in the reverse reaction; both reactions require either Mg<sup>2+</sup> or high concentrations of monovalent ions to proceed (16). Like other naturally occurring ribozymes, the HPR may be relic of the RNA World (1), and freezing-induced ligation has several features that would have been helpful for development of the RNA World. Ligation provides a means for increasing RNA length and complexity by combining short fragments made by other processes. At lower temperatures, fewer intermolecular interactions (such as hydrogen bonds) would be required to stabilize complexes, favoring the formation of ribozymes from small RNA fragments. Also, a pair of fragments could potentially be ligated without requiring extensive base-pairing with the ribozyme, reducing the need for specific sequence complementarity. Moreover, freezing-induced concentration could contribute to ligation efficiency by bringing together diluted RNA fragments to form catalytically active complexes. And most important, low temperatures would also greatly limit degradation of the newly formed RNA molecules. In later stages of molecular evolution, the larger, more stable, and efficient catalysts may have survived transport into more ‘warm and wet’ environments by virtue of their synthetic power outpacing degradation.

To test the hypothesis that freezing temperatures could support RNA synthetic reactions by primitive ribozymes, we decided to use a ‘reverse-evolution’ approach. This approach

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involves progressive minimization, fragmentation and deletion-substitution mutagenesis of the original, naturally existing ribozyme in order to access related sequences that might have included its 'ancestors'. Following this strategy, the HPR was simplified by eliminating loops, shortening helical segments, eliminating connecting sequences between the catalytic core and the substrate binding domain, and finally deleting the substrate binding domain altogether. Each construct was assayed for the ability to ligate pairs of RNA fragments with defined or randomized sequences. In addition, we tested how a critical point mutation introduced in the HPR catalytic core affects its catalytic activity under both solution and freezing conditions. It was hoped that this approach would reveal examples of the sort of primitive ribozymes that could have promoted the evolution of RNA complexity in protected, low-temperature environments.

## MATERIALS AND METHODS

### Ribozymes

A 57-nt HPR (structure I, Fig. 7) was generated from a T7 promoter sequence by *in vitro* transcription of a DNA template strand of the sequence 5'-TACCAGGTAATGTACCACGACTTACGTCGTGTGTTTCTCTGGTTGACTTCTCTGCCCTATAGTGAGTCGTATTA-3' (IDT; promoter underlined).

For the bisected ribozyme (structure II), the 5' part (30nt) was transcribed from the DNA template 5'-CGTGTGT-TTCTCTGGTTGACTTCTCTGCCCTATAGTGAGTCGTA-TTA-3' and the 3' part (19 nt) was the synthetic RNA 5'-CGUGGUACAUAUACUGGUA-3' (Dharmacon).

The HPR with deleted terminal stem and loop (structure III) was transcribed from the DNA template 5'-TACCAGGT-AATGTACCGTGTTTCTCTGGTTGACTTCTCTGCCCTA-TAGTGAGTCGTATTA-3'.

For the ribozymes with separated domains (structures IV and V), the 3' part (42 nt) was transcribed from the DNA template 5'-GGGAGGTAATGTACCACGACTTACGTCG-TGTGTTTCTCTCCCTATAGTGAGTCGTATTA-3' and the 5' parts (14 or 10 nt) were the synthetic RNAs 5'-GGGCAGAGAAGUCA-3' or 5'-AGAGAAGUCA-3', respectively. The HPR with deleted substrate-binding domain (structure VI) was identical to the 42 nt 3'-part of structures IV and V.

Individual RNA species obtained by *in vitro* transcription or chemically synthesized were mixed with 3× denaturing loading solution (7 M urea, 10 mM EDTA, 0.02% xylene cyanol and 0.02% bromophenol blue) and purified by electrophoresis on 15% polyacrylamide gels containing 7 M urea. RNA bands were localized by UV shadowing and isolated by excising bands from the gel, crushing and extracting the RNA in 0.3 M sodium acetate pH 5.0. After ethanol precipitation, the samples were dissolved in 5 mM Tris-HCl pH 7.5 and stored at -20°C.

### Ligation substrates

Ligation substrates 3'-LS and 5'-LS (Fig. 1) were obtained by HPR-catalyzed cleavage of an internally <sup>32</sup>P-labeled oligonucleotide (LP), generated by T7 promoter-driven *in vitro* transcription from a DNA template strand of the sequence 5'-CACAGTCTAGTCGTCAGCAGGGGCAGG-

ACTGTCAGACGCATACTTGCTAGACTGCCCTATAGT-GAGTCGTATTA-3'. LP consisted of the normal HPR substrate sequence extended with flanking sequences for use as primer binding sites for selection experiments (see Fig. 5). After cleavage was performed in 10 mM MgCl<sub>2</sub>, 1 mM Tris-HCl (pH 7.5) at 37°C, the two products were gel purified as described above.

### Reaction procedures and analysis of the products

The freezing reactions were performed essentially as described by Kazakov *et al.* (15). Typical 10 µl reactions contained ~0.3 µM internally <sup>32</sup>P-labeled ligation substrates 5'-LS and 3'-LS and unlabeled ribozyme core in 25 mM NaCl, 1 mM Tris-HCl (pH 7.5). To prevent variation in the rate of freezing we used a modified quick-freezing technique (17). Ten microliter samples in 1.5 ml Eppendorf microcentrifuge tubes were frozen by immersing in a dry ice bath (-79°C) for 3 min, followed by transfer to an ethanol-water bath (MGW Lauda RC3 Model B-2, Brinkman) and incubation at the desired temperature (typically -10°C).

The reaction was stopped by removing tubes at the indicated time intervals, mixing with 3× loading buffer, and analyzing on denaturing 15% polyacrylamide gels. <sup>32</sup>P-labeled reaction products were quantified using a Molecular Imager FX and Quantity One-4.2.0 software (Bio-Rad). Apparent initial rates for the freezing-induced ligation reaction were determined from the initial slope of the plots. The fraction of substrate ligated per min was calculated using the 5–15 min time interval (three initial points).

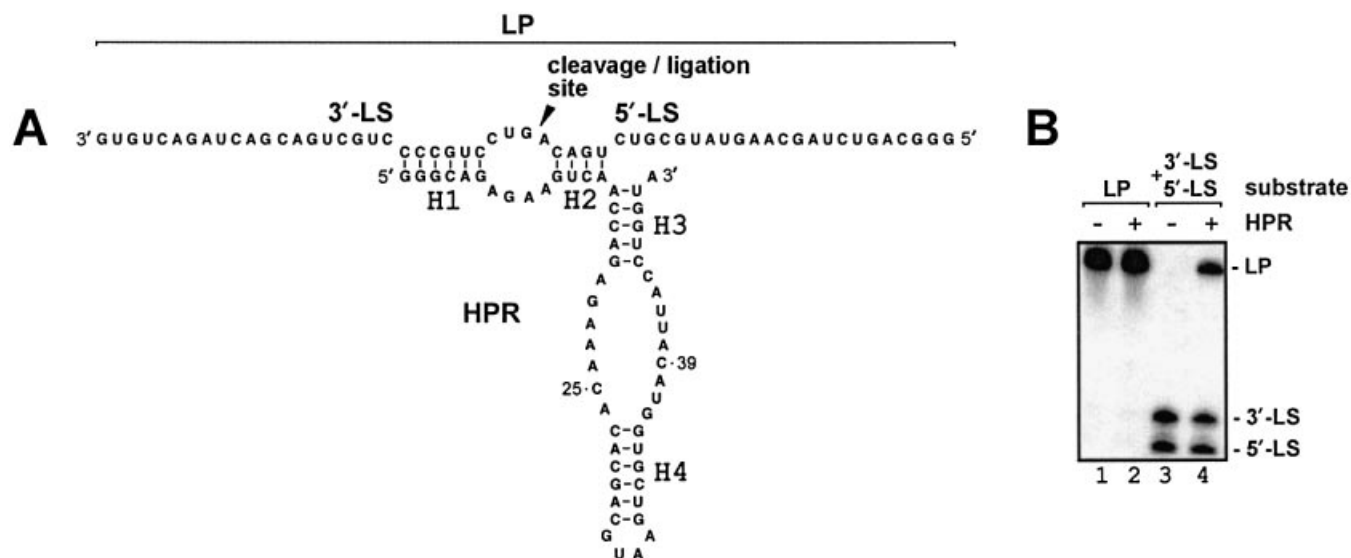
### Selection of substrates suitable for HPR ligation under freezing conditions

Two selection experiments were performed. In one, the 3'-LS was partially randomized at nine residues, six of which normally should form base pairs with the substrate binding sequence of the HPR (helix 1): 5'-NNNNNNNNNCUGCUGACGACUAGACUGUG-3' (3'-LS-N, Fig. 5A), and used for ligation with the 5'-LS. In the second selection experiment, the 5'-LS was partially randomized at five residues, four of which normally should form base pairs with the substrate binding sequence of the HPR (helix 2): 5'-GGGCAGUCUAGCAA-GUAUGCGUCNNNNN-3' (5'-LS-N, Fig. 5B), and used for ligation with the 3'-LS. Ligation substrates were incubated with HPR at -10°C for 14 h, products of the ligation reaction were reverse transcribed, PCR amplified, cloned and sequenced.

## RESULTS

### Freezing-induced HPR ligation *in trans*

To determine whether the freezing-induced catalysis previously seen for HPR ligation *in cis* (intramolecular reaction) (15) also occurred *in trans* (intermolecularly), we examined the *trans*-acting version of the HPR shown in Figure 1A with bound substrates. Freezing experiments were performed in 25 mM NaCl, 1 mM Tris-HCl pH 7.5, which we made standard conditions for the freezing reactions. Solutions containing unlabeled HPR and <sup>32</sup>P-labeled ligation substrates 5'-LS and 3'-LS were quick-frozen and then incubated at -10°C. For comparison, reactions were also performed under



**Figure 1.** (A) Structure of the hairpin ribozyme with separated enzyme and ligation substrates. Helical segments H1–H4 as well as ligation substrates 3'-LS and 5'-LS and the cleavage/ligation site are as indicated. The HPR used in this study differed from the canonical sequence in having additional stabilizing GC base pairs in Helix 4 capped with a tetraloop structure and a U39→C substitution since both modifications are known to improve HPR activity (19). Ligation substrates 3'-LS and 5'-LS were obtained by HPR-catalyzed cleavage of an internally  $^{32}\text{P}$ -labeled oligonucleotide (LP), consisting of the normal HPR substrate sequence extended with flanking sequences for use as primer bindings sites for selection experiments (see Fig. 5). After cleavage was performed in 10 mM  $\text{MgCl}_2$ , 1 mM Tris–HCl (pH 7.5), at 37°C, the cleavage products were gel purified. (B) HPR catalyzed ligation *in trans* under freezing conditions. Lanes 1 and 2, cleavage substrate (identical to ligation product, LP) incubated with (+) or without (–) HPR. Lanes 3 and 4, ligation substrates (3'-LS and 5'-LS) incubated with (+) or without (–) HPR. Reactions were performed at a 1:1 ratio of 3'-LS to 5'-LS and 20:1 HPR-to-substrate ratio, in 25 mM NaCl, 1 mM Tris–HCl (pH 7.5), –10°C, 14 h.

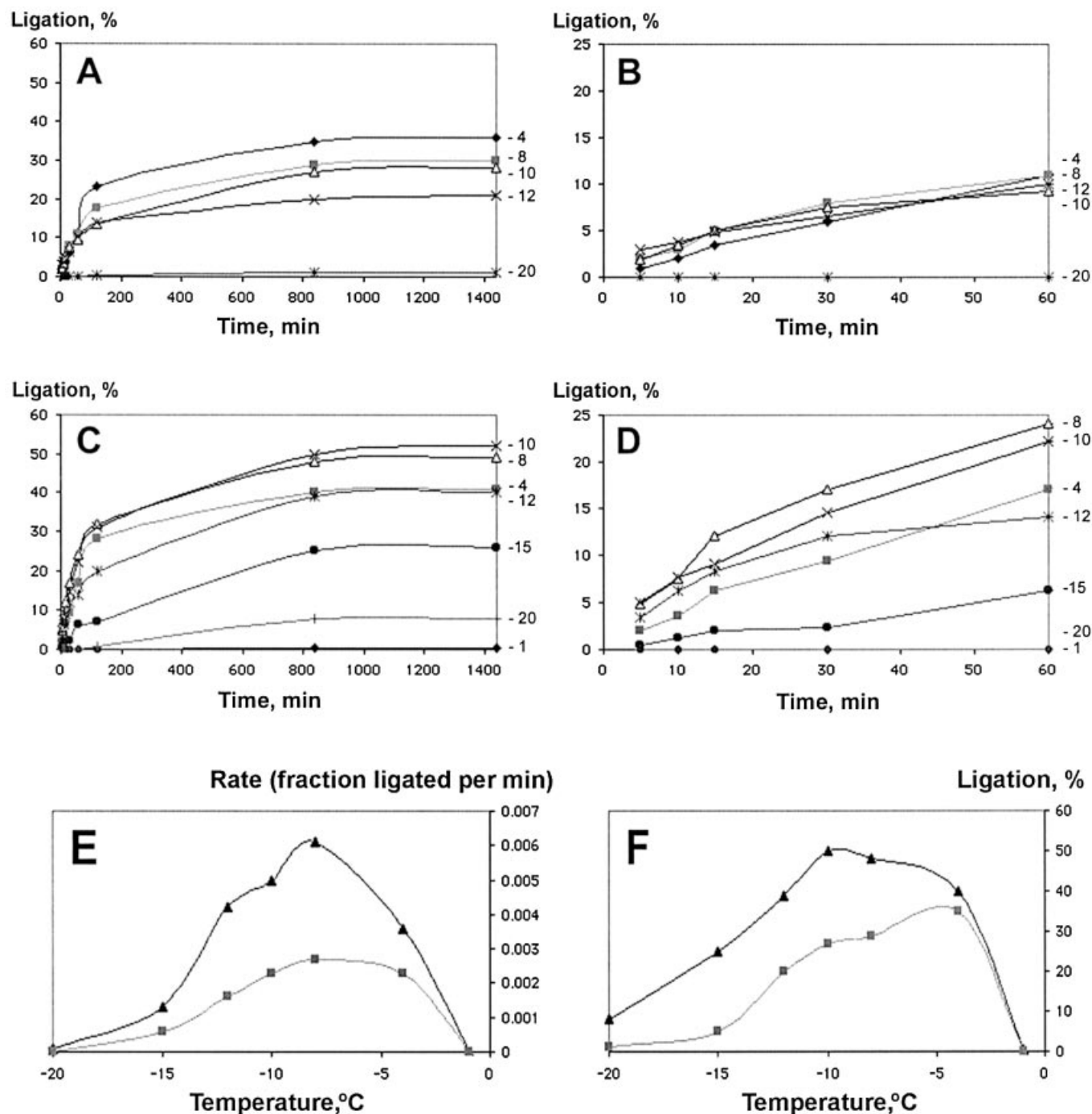
normal solution conditions: 10 mM  $\text{Mg}^{2+}$ , 50 mM Tris–HCl pH 7.5, 37°C (18,19). We indeed observed formation of the expected ligation product. Freezing-induced ligation is much more efficient than cleavage: after 14 h at –10°C, the ligation yield reached 50% whereas cleavage of the full-length substrate was only 0.5% (Fig. 1B). A longer than 14 h incubation did not provide higher yields for either reaction (see below), and thus this time point was taken as representing equilibrium in what follows. However, in equilibrium, HPR cleavage and ligation percentages should add up to 100%. We attribute the fact that the yields of these two reactions do not add up to 100% to a significant fraction of the ribozyme population being in an inactive conformation at low temperature. Similar, but milder, conformational trapping of HPR has been also observed in normal solution reactions (20,21).

The kinetics of ligation of equimolar amounts of the two substrates and a 1:1 or 20:1 ribozyme-to-substrates ratio for various sub-zero temperatures are shown in Figure 2A–D. Although ligation is efficient at the 1:1 HPR-to-substrates ratio, elevating the ratio to 20:1 increases the ligation yield (compare Fig. 2A and C). The beneficial effect of excess ribozyme further supports the suggestion that some HPR molecules become trapped in inactive conformations, although there may also be some contribution from an increase in the fraction of substrates bound to ribozyme. This observation also indicates that even at freezing temperatures the substrates can easily dissociate from incomplete complexes with the ribozyme (HPR: 3'-LS or HPR: 5'-LS) and reassociate to form complete complexes (HPR: 3'-LS: 5'-LS).

If the solution was supercooled to –10°C without freezing, no ligation occurred (data not shown). This indicates that

freezing is absolutely required for the reaction and suggests that the dehydrating effects of freezing induce the catalytically active conformation independent of the presence of divalent metal ions (15). In contrast to the onset of catalytic activity when the solution freezes, in earlier studies describing HPR in solutions, activity was seen to decrease with temperature (22,23). When we performed control reactions in  $\text{Mg}^{2+}$ -containing buffer at 37°C, ligation was inefficient, with a yield of only ~4% after 14 h, whereas the LP substrate was over 90% cleaved. Thus, for the *trans*-HPR system used, the cleavage-ligation equilibrium is shifted towards ligation under freezing conditions and towards cleavage at 37°C. This difference is presumably because the products of cleavage remain largely bound to the ribozyme at low temperatures but not at 37°C. Indeed, stabilization of the HPR-substrate complex (increasing the number of base pairs, low temperature) is known to shift equilibrium towards ligation, while destabilization favors cleavage (22).

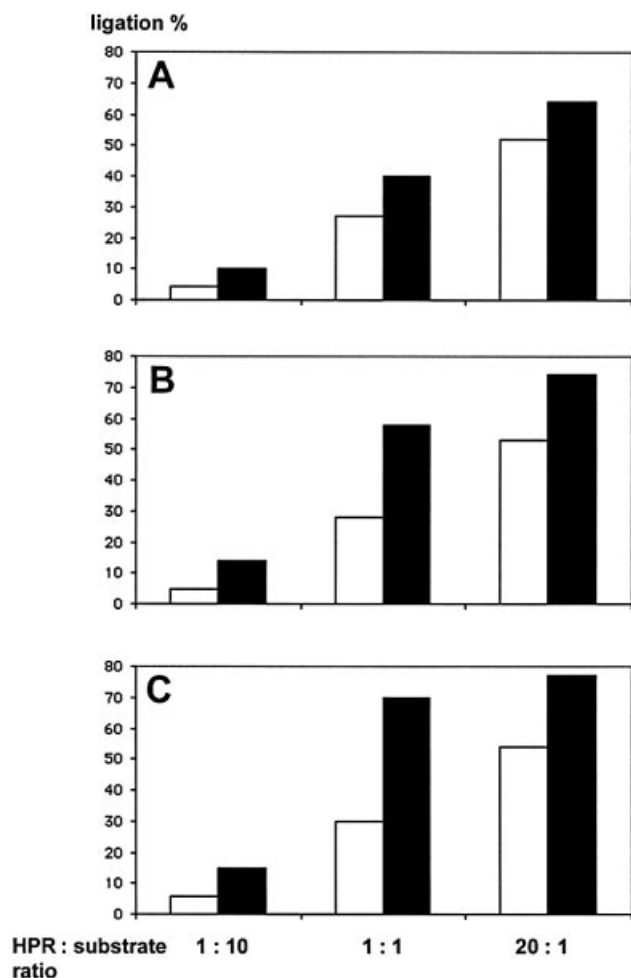
The temperature dependence of the yield and apparent initial rate of the freezing reaction is shown in Figure 2E–F, based on data from Figure 2A–D. In these experiments a 1:1 ratio of the ligation substrates 3'-LS and 5'-LS was maintained, and the yield was calculated for the 14 h time-point based on Molecular Imager quantitation of polyacrylamide gel separations. Apparent initial rates were determined from the initial slope of the plots (see Materials and Methods). The reaction is most efficient in the range –4° to –12°C, with rates dropping to near zero for temperatures above –1° or below –20°C. Thus, although the reaction requires freezing, the reaction temperature cannot be too cold, presumably due to the need for sufficient thermal energy to surmount the



**Figure 2.** (A–D) Kinetics of the HPR-catalyzed ligation *in trans* at different temperatures (shown at the right). (A) Ligation of equimolar amounts of the 3'-LS and 5'-LS, at HPR-to-substrate ratio 1:1, 5 min to 24 h; (B) the enlarged initial 5–60 min time interval for (A). (C) Ligation of equimolar amounts of 3'-LS and 5'-LS, at HPR-to-substrate ratio 20:1, 5 min to 24 h; (D) the enlarged initial 5–60 min time interval for (C). Average values from three experiments are shown. (E–F) Initial rates (E) and ligation yields (F) for HPR-catalyzed ligation *in trans* at different temperatures, based on data presented in (A–D). HPR-to-substrate ratios 20:1 (triangles) or 1:1 (squares).

activation barrier for the reaction, and the presence of the solution phase as microinclusions in ice. In the prebiotic environment optimal conditions for the reaction would probably include temperatures slightly below freezing, with fluctuations creating frequent cycles of freezing and melting, as occurs for example in partially frozen pools of water. Melting and warming of frozen solutions would result in

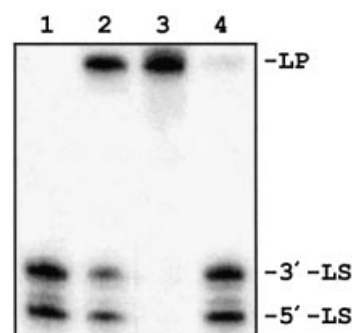
dissociation and redistribution of primordial ribozyme and ligation substrates as well as in refolding of the ribozymes. We mimicked these cycling conditions to see if they could affect ligation yields. Samples were frozen at  $-10^{\circ}\text{C}$  for 14 h, melted and incubated at  $37^{\circ}\text{C}$ , then frozen again, with the procedure repeated two to six times. Freeze–thaw cycles significantly increased the ligation yields. The effect was largest when the



**Figure 3.** Effect of freezing/melting cycles on the yield of ligation reaction. White bars: samples incubated continuously at  $-10^{\circ}\text{C}$  for (A) 28 h, (B) 56 h and (C) 84 h. Black bars: the samples were frozen at  $-10^{\circ}\text{C}$  for 14 h, melted and incubated at  $37^{\circ}\text{C}$  for 5 min, then frozen again, with the procedure repeated (A) two times, (B) four times and (C) six times.

substrates were in 10-fold excess over the HPR, in which case cycling tripled the yield (from 5 to 15%) relative to non-cycled control samples (Fig. 3). This indicates a turnover number of 1.5 (0.15/0.1) for the ribozyme, consistent with release of the ligated product at the higher temperature to allow new substrates to bind. Like the effect of excess ribozyme, the effect of cycling is consistent with only a fraction of the HPR being active and the rest trapped in inactive conformations. If the active molecules are stabilized by binding to substrate and product, freeze-thaw cycles could also be 'pumping' inactive ribozymes into the active confirmation.

To determine the nature of the linkage formed by the HPR upon ligation under freezing conditions, the 3'-LS and 5'-LS were ligated by the HPR at  $-10^{\circ}\text{C}$ . The ligation product was gel-purified and incubated with the HPR in Mg-containing buffer at  $37^{\circ}\text{C}$  to allow cleavage to proceed. If linkage is 2'-5', no cleavage should occur, since the HPR is known to cleave exclusively 3'-5' bonds under standard solution conditions (24). Since the substrate obtained by ligation was recleaved essentially to completion (Fig. 4), we conclude that a natural



**Figure 4.** HPR forms mainly or exclusively 3'-5' linkage upon ligation under freezing conditions. Lane 1, ligation substrates 3'-LS and 5'-LS obtained by HPR cleavage under standard solution conditions ( $37^{\circ}\text{C}/10\text{ mM Mg}^{2+}$ ) and gel-purified. Lane 2, ligation of the 3'-LS and 5'-LS by the HPR under freezing conditions ( $-10^{\circ}\text{C}$ ), 14 h. Lane 3, gel-purified ligation product LP. Lane 4, cleavage of the LP by the HPR under standard solution conditions ( $37^{\circ}\text{C}/10\text{ mM Mg}^{2+}$ ), 2 h.

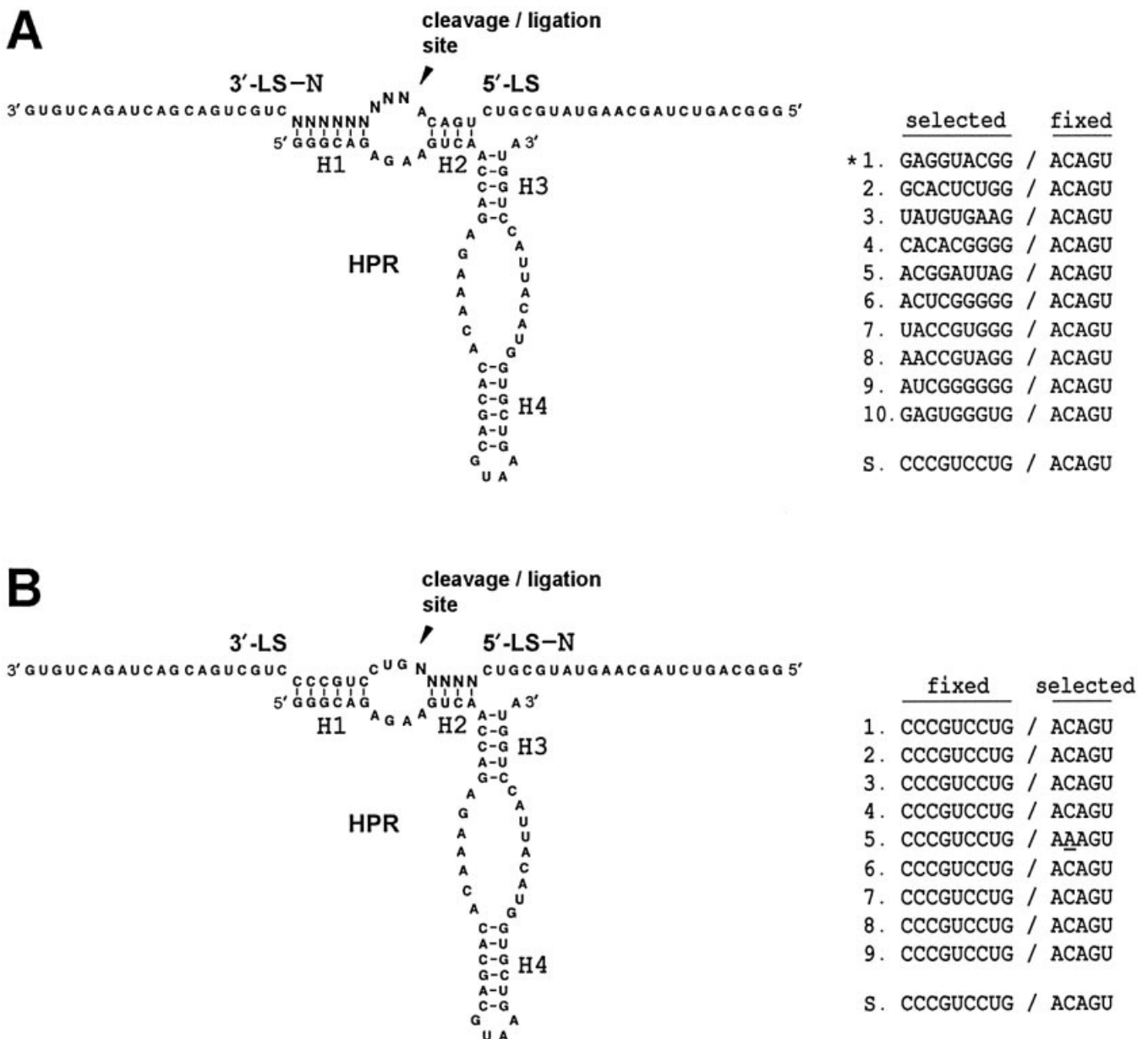
3'-5' phosphodiester bond was the principal product of HPR-catalyzed ligation.

Varying the NaCl concentration in the range 10–100 mM had no effect on the freezing-induced ligation *in trans*. The presence or absence of  $\text{Mg}^{2+}$  (0–10 mM) also made no difference, consistent with previous observations showing that  $\text{Mg}^{2+}$  is not required for freezing-induced ligation *in cis* (15). Other studies on the solution HPR reaction, showing that divalent metal ions can be substituted by cobalt (III) hexamine, spermidine or high concentrations of monovalent cations such as  $\text{Na}^+$  (22,25–30), also support only a structural but not catalytic role for divalent metal ions. Increasing the total RNA concentration by 5-fold had no effect on the freezing-induced *trans*-ligation, indicating that the rate-limiting step is not affected by freezing induced concentration under the conditions tested.

We anticipated that slow freezing or pre-incubation of the reaction mixture at  $4^{\circ}\text{C}$  prior to quick-freezing might facilitate formation of substrate-ribozyme complexes and thus increase ligation efficacy. Surprisingly, these procedures produced the same results as the standard method of quick freezing. Moreover, samples quick-frozen in liquid nitrogen ( $-170^{\circ}\text{C}$ ) and in dry ice ( $-80^{\circ}\text{C}$ ) gave similar ligation yields. The relative insensitivity to environmental conditions as long as freezing occurs underscores the robustness of the system and increases the likelihood that conditions on the prebiotic earth could have supported freezing-induced ligation.

### Sequence requirements of freezing-induced ligation

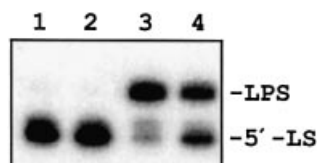
At lower temperature, fewer base pairs should be required for stable pairing between the ribozyme and the substrate sequences, suggesting that the sequence specificity of the ligation reaction may be reduced for freezing-induced catalysis compared to the normal solution reaction. To test whether this is indeed the case, the 3'-LS was partially randomized at nine residues, six of which normally should form base pairs with the substrate binding sequence of the HPR (helix 1). The resulting 3'-LS-N (5'-NNNNNNNNN-CUGCUGACGACUAGACUGUG-3', Fig. 5A) was used in the freezing ligation reaction at  $-10^{\circ}\text{C}$  with the 5'-LS



**Figure 5.** Selection of substrates suitable for HPR ligation under freezing conditions. Experimental scheme and substrate sequences selected in experiments with (A) randomized 3'-ligation substrate (3'-LS-N), and (B) randomized 5'-ligation substrate (5'-LS-N). Ligation substrates were incubated with the HPR at  $-10^{\circ}\text{C}$  for 14 h. Products of the ligation reaction were reverse transcribed, PCR amplified, cloned and sequenced. S, standard sequence (consensus). Sequence 1 (marked with asterisk) was chemically synthesized and tested for ligation.

(5'-GGGCAGUCUAGCAAGUAUGCGUCUGACA-3'). Products of the reaction were reverse transcribed and PCR-amplified using the underlined sequences as primer-binding sites. Cloning and sequencing revealed that 3'-substrate sequences ligated by the HPR all contained a G at the 5'-end where ligation occurred, as is known to be absolutely required for catalysis by the wild-type HPR (31) (Fig. 5A). However, all other selected positions were variable, with no sequence in common among clones sequenced. When the ligation mixture was analyzed directly on the gel without RT-PCR, the products of ligation were readily visible and represented up to 2% of the 3'-LS-N pool. Thus the 5'-LS-ribozyme core complex appeared to be capable of ligating essentially any

sequence having a 5'-terminal G [and even the requirement for a terminal G might be eliminated if the C at position 25 is mutated (32)]. In contrast, when a similar selection was performed with the 5'-LS-N randomized at five 3'-terminal residues, four of which normally should form base pairs with the substrate binding sequence of the HPR (helix 2), most of the successfully ligated sequences matched the original 5'-LS sequence (Fig. 5B). Thus, under the freezing conditions four base pairs are required to bind the 5'-LS but virtually any sequence can be ligated by the HPR-5'-LS complex. To confirm the validity of this conclusion, we synthesized the 3'-LS substrate sequence 1 (Fig. 5A), which has no complementarity to the substrate-binding domain. This substrate was



**Figure 6.** HPR-catalyzed ligation of the short version of the 3'-ligation substrate (GUCCU) with the 5'-LS under freezing versus standard solution conditions. Lanes 1 and 2, standard solution conditions (37°C/Mg<sup>2+</sup>). Lanes 3 and 4, freezing conditions (−10°C). Ligation substrates were used in equimolar amounts, and the ribozyme-to-substrate ratio was 20:1 (lanes 1, 3) or 1:1 (lanes 2, 4). 5'-LS was internally <sup>32</sup>P-labeled, 3'-ligation substrate unlabeled. LPS, ligation product.

efficiently ligated to 5'-LS at −10°C (25% yield after 14 h). As expected, no ligation was seen after 14 h incubation at 37°C in the presence of Mg<sup>2+</sup>. Similar results were obtained with a rationally designed 5 nt sequence capable of forming just two out of the canonical 6 bp forming the HPR helix 1 (Fig. 6).

The finding of reduced substrate specificity in the freezing reaction compared to the normal solution reaction reinforced the idea that the relative strengthening of weak interactions at low temperatures could support catalysis by complexes of HPR fragments that would fall apart at higher temperatures. In order to test the hypothesis that primitive catalytic structures can be assembled at sub-zero temperatures through weak interactions, we tested a number of fragmented and truncated versions of the HPR (Fig. 7). The substrates used for ligation were the same as described above, 3'-LS and 5'-LS (Fig. 1A). We found that bisecting the RNA of the catalytic core by cutting the loop at the bottom of helix 4 (structure II) reduced the yield of ligation at −10°C by about half, from 50 to 23%, and reduced the ligation yield at 37°C in the presence of Mg<sup>2+</sup> from 4 to 1% in all standard 14 h reactions. Eliminating helix 4 and connecting the C and G that would otherwise form its first base pair (structure III) had a severe effect, reducing the yield of the freezing reaction to 0.7%. Separating the catalytic core from the substrate-binding domain of I (structure IV) reduced the ligation yield from 50 to 10%, and decreasing the length of the sequence that participates in helix 1 from 6 to 2 nt (structure V) further reduced the yield to 1%. These results are consistent with earlier reports (33,34) that the cleavage activity of the separate-domain HPR is much lower than that of the bisected HPR and intact HPR. Interestingly, the complete elimination of the substrate binding sequence (structure VI) still left detectable ligation activity under freezing conditions (0.3%, compared to background of 0.05%); however, in Mg<sup>2+</sup>-containing solution at 37°C, structure VI (as well as III–V) had no detectable activity (≤0.05%). It is unclear whether this untemplated ligation results from non-specific binding of the substrates to the catalytic core, or from substrate sequences playing the role of the substrate-binding domain. In the latter case, with low temperature stabilizing weak interactions, one of the substrates might act as a highly mismatched splint to position a separate pair of 3'-LS and 5'-LS substrates for ligation by the catalytic core.

Finally, an HPR variant in which G8 is substituted with U (HPR:mutG8→U) was assayed for ligase activity under freezing conditions. Substitution of G8 by any other nucleotide is known to completely abolish ribozyme activity under

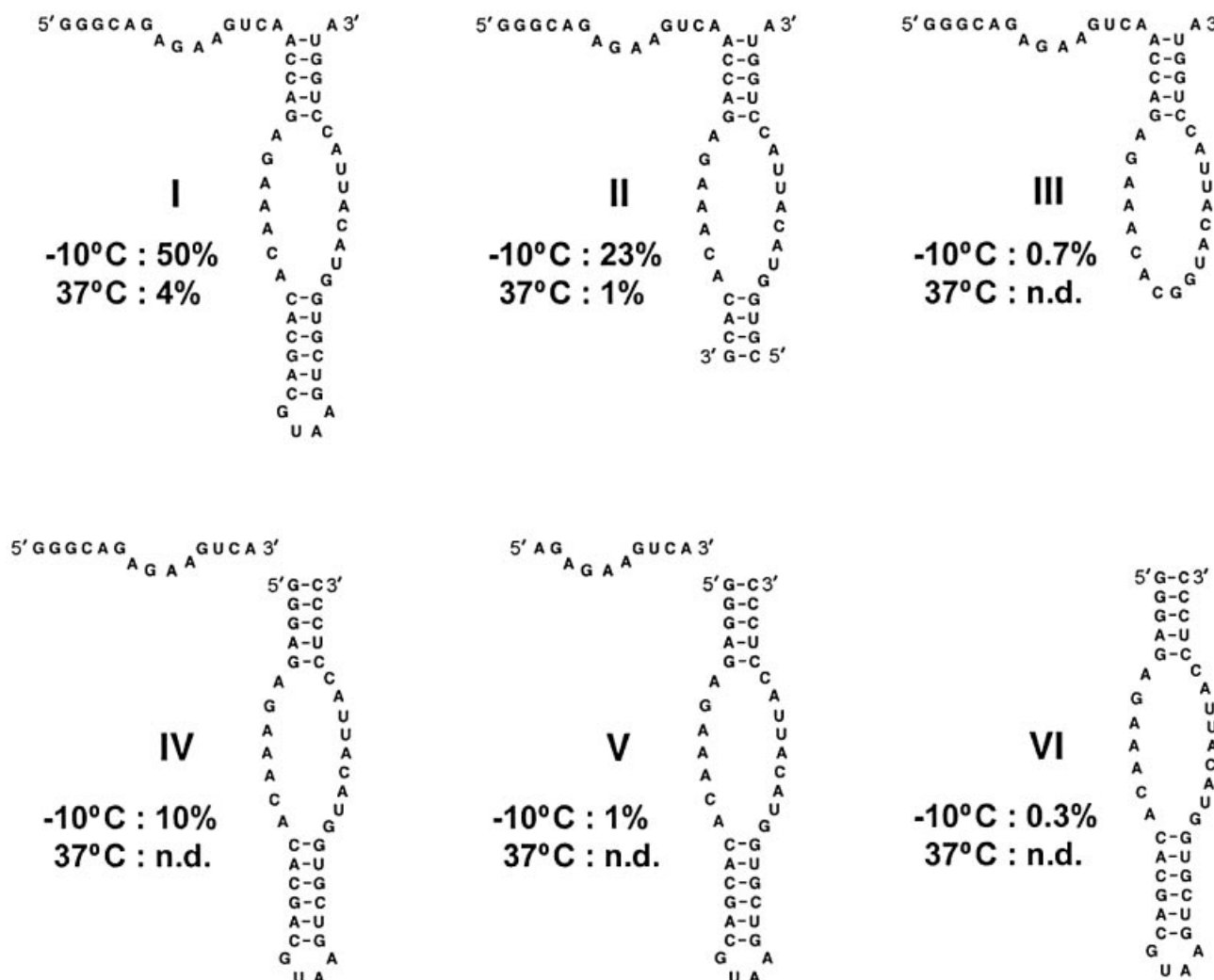
standard solution conditions (37°C/Mg) (35). Indeed, in control experiments at 37°C, neither cleavage nor ligation activity were detectable. However, under freezing conditions this mutant was active, with ligation yields of 5.6% at ribozyme-to-substrate ratio 1:1, in a standard 14 h reaction. As in the case of the parental HPR, increasing the ribozyme-to-substrate ratio to 20:1 led to a moderate increase in reaction yields. Taken together, our results show that freezing is uniquely able to relax the sequence and structure requirements for the HPR. Freezing allows mutated, partially deleted and fragmented HPR derivatives to be efficient catalysts at sub-zero temperatures despite having greatly reduced or zero activity in solution.

## DISCUSSION

The results presented build on earlier findings that freezing provides conditions that allow efficient *cis*-ligation (circularization) of the HPR in the absence of the usual levels of metal ions required to support the solution reaction (15). The lack of dependence on magnesium or other divalent cations is consistent with evidence that such ions do not play a catalytic role in the normal solution reaction for this ribozyme (36). We now find that freezing supports efficient ligation *in trans*, with the cleavage-ligation equilibrium strongly favoring ligation. By comparison, the equilibrium constant for the solution reaction in 50 mM Tris–HCl, 10 mM MgCl<sub>2</sub> increasingly favors ligation with lower temperature down to 20°C (22). Lowering of temperature reduces the reaction rate as expected (22,23); it becomes negligible below 0°C and no ligation was seen for samples that were supercooled but not frozen. As seen in Figure 2, the onset of freezing dramatically increases the rate of ligation.

By investigating the sequence requirements for ligation *in trans*, we find that sequence specificity (i.e., the requirement for base pairing between substrates and ribozyme) is greatly reduced for the freezing reaction compared to the solution reaction. The fact that weak interactions are stabilized at lower temperatures doubtless accounts for much of this effect, but factors specific to freezing such as increased solute concentration and lowered water activity may also contribute to altered specificity. The ability to ligate a wide variety of fragments with little or no regard for sequence provides an important missing link in the prebiotic RNA world scenario: a mechanism for creating a pool of high-complexity genetic raw material for subsequent selection. Replication of selected sequences requires a copying mechanism with reasonable fidelity, but at the level of forming the high-complexity pool there need be no such constraint.

Under freezing conditions the HPR can be extensively truncated, partitioned into separate RNA fragments and mutated while still retaining the ability to ligate a wide variety of sequences together. Pinard *et al.* (37) have argued for a catalytic role for G8, which is critical for the standard solution reaction. However, the fact that G8 can be substituted by U in the freezing reaction argues against a catalytic role for this residue. The truncated versions are of the order of size of the smallest ligating ribozyme described to date, 29 nt, which was obtained through *in vitro* selection experiments (38). The ligation rates of the fragmented HPR are not high compared to the parent ribozyme, but the reaction can be promoted by



**Figure 7.** HPR derivatives with ligation yields observed after 14 h incubation at  $-10$  and  $37^{\circ}\text{C}$ . Ligation substrates 3'-LS and 5'-LS were used in equimolar amounts, and the ribozyme-to-substrate ratio was 20:1. I, HPR; II, bisected HPR; III, truncated HPR; IV and V, HPR with separated domains; VI, template-deleted HPR.

freeze-thaw cycling such as would be provided by day-night temperature fluctuations. From an evolutionary point of view, all that is needed is that ligation rates exceed degradation. The fact that the ligation yield was never seen to exceed  $\sim 75\%$  despite the fact that cleavage is nearly undetectable suggests that some enzyme-substrate complexes are trapped in unproductive structures. The ability of freeze-thaw cycling to drive the reaction to somewhat higher efficiency supports this notion, but indicates that such cycling can only rescue some of the unproductive complexes. In a similar vein, changes in the rate of cooling of the reactants prior to incubation had no significant effect on the reaction.

The results presented here provide a mechanism for RNA to increase in complexity in the prebiotic environment through ligation of smaller RNAs, which is attractive compared to step-by-step polymerization (39). The ability of RNA to increase in complexity while being protected from degradation by sub-zero temperatures provides new support for cold-origins theories of prebiotic evolution. Also, the ability of RNA to catalyze synthetic reactions under conditions where it

is chemically stable could have applications in biotechnology. However, a thorough understanding of freezing-induced catalysis will require much additional study. Several basic questions remain unanswered, such as whether the reaction takes place entirely in the liquid phase or involves contributions from the ice-liquid interface, and whether concentration plays a role [the efficiency of the HPR ligation *in cis* reaction (15) argues that it does not].

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