Effect of Unpaired Terminal Nucleotides of Substrate RNAs on the RNA Ligation with T4 RNA Ligase

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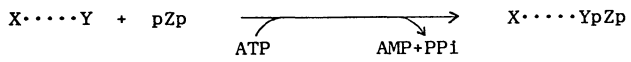
The effect of double-helix formations and unpaired terminal nucleotides of RNA oligomer substrates on a RNA ligation with T4 RNA ligase has been investigated by using high-performance liquid chromatography (HPLC), UV absorbance, and circular dichroism (CD) measurements, and the energy calculation for the double-helix stability of RNAs. In the 1-hour reaction of single-stranded RNA acceptors, the yield of the product increased in the order of pyrimidine donors pCp>pUp and purine donors pAp>pGp. In the reaction of a double-stranded RNA acceptor, UGCGCA, the yield also increased in the same order of the donors. On the other hand, in the reaction of double-stranded RNA acceptors containing unpaired terminal nucleotides (dangling ends), pUp and pGp donors gave the largest yields of the products in the reactions of AUGCGCA and CAUGCAU acceptors, respectively. Furthermore, the yield of the product is the largest for CAUGCAU acceptor within the acceptors having A, C, G, and U dangling ends in the reaction of pGp donor. These results sugggest that the formation of the terminal base pairs of the donors and the dangling ends may affect the RNA ligation with T4 RNA ligase.

Unpaired terminal nucleotides (dangling ends) play very important roles in the structure and interaction of ribonucleic acids (RNAs). It is well-known that the dangling ends determine the stability of codon–anticodon associations[1,2] and are responsible for some codon context effects.[3] It is also known that the nucleotides determine important tertiary structures of a transfer RNA (tRNA)[4] and a catalytic core of *Tetrahymena* ribozyme.[5,6] Recently, the effect of the dangling ends on the stability of RNA duplexes has been studied energetically.[7,8] The result showed that both 3′ and 5′ terminal nucleotides stabilized the core duplexes. However, little is known about the effect of the unpaired terminal nucleotides on biochemical reactions such as ligase and nuclease reactions.

In this work, we have investigated the effect of the unpaired terminal nucleotides of substrate RNAs on the RNA ligation with T4 RNA ligase of which the overall reaction is shown in Scheme 1. The study can provide insight into the contribution of the formation of a substrate double-helix and terminal base-pair to the RNA ligation.



X,Y,Z : rA,rC,rG, or rU
p : phosphate

Scheme 1.

Experimental

Materials. UGCGCA, AUGC, AUGCA, AUGCG, and XAUGCAU (X=A, U, G, C) were synthesized chemically on a solid support with phosphoramidite procedures[9] and purified with high-performance liquid chromatography (HPLC) after deblocking. AUG was purchased from Sigma Chemical Co. These oligomers were further purified and desalted with a C-18 Sep-Pak cartridge. Final purity of the oligomers was checked by HPLC and was greater than at least 95%. The concentration of the oligomers was determined as described previously.[10]

Nucleotides 3′,5′-bisphosphates (pZp), albumin from bovine serum (BSA), and T4 RNA ligase were purchased from Pharmacia, Sigma Chem. Co., and BRL Inc., respectively. Adenosine 5′-triphosphate (ATP) and dithiothreitol (DTT) were products of Wako Chem. Ind.

T4 RNA Ligase Reaction. The RNA ligase reaction mixture contained 2×10^{-1} mol dm^{-3} Tris-HCl (pH 7.5), 2×10^{-2} mol dm^{-3} $MgCl_2$, 7.5×10^{-3} mol dm^{-3} ATP, 5×10^{-3} mol dm^{-3} DTT, 25×10^{-9} g dm^{-3} BSA, 1×10^{-3} mol dm^{-3} acceptor RNA oligomer, and 25 to 90 unit/ml (U/ml) of the enzyme. At the assay for the reaction, 4×10^{-3} mol dm^{-3} donor pZp was also present in the reaction mixture. After incubation for adequate times, mainly for 1 h at 37 °C, the reaction mixture was heated for 2 min at 100 °C and treated with alkaline phosphatase for 20 min at 65 °C.[11]

The analysis was performed by HPLC using a Tosoh LC System and a Cosmosil Packed Column 5C18-AR onto which aliquots from the reaction mixtures were injected. The analysis was done at a flow rate of 1 ml min^{-1} with a 50-min linear gradient from 0 to 25% methanol containing 10^{-1} mol dm^{-3} NaH_2PO_4. The peaks at 254 nm corresponding to the substrates, intermediates, and products were integrated using a Tosoh Sic Chromatocorder 12. The extent of reaction was determined by the ratio of the peak area of the product to that of the substrate. The HPLC analysis indicated no significant loss of the substrate due to contaminating nucleases.

Spectroscopy. UV absorbance spectra of the RNA oligomers were measured on a Tosoh UV 8011 spectrophotometer. Their UV melting curves (absorbance vs. temperature curves) were measured on a Hitachi U-3200 programmable spectrophotometer equipped with a Hitachi SPR-7 temperature controller. The heating rate was 0.5 or 1 °C min^{-1}. A melting temperature, T_m, at each oligomer concentration of 10^{-3} mol dm^{-3} was determined by Eq. 1:[12]

$$T_m = \Delta H^\circ / (\Delta S^\circ + R \ln(C_t)), \quad (1)$$

where ΔH° and ΔS° were the enthalpy and entropy changes for the double-helix formation of the oligomer,[7] R was the gas constant, and C_t was 10^{-3} mol dm^{-3}. Circular dichroism (CD) spectra of the oligomers were measured on a JASCO J-600 spectropolarimeter equipped with the temperature controller.

Stability Calculation of RNA Duplexes. The stability energy of the oligomer duplexes was obtained from nearest-neighbor thermodynamic parameters[7,12] as described previously.[13] For example, the entropy change ΔS° for the formation of the self-complementary UGCGCA duplex was calculated as follows:

$$\begin{aligned}\Delta S^\circ &= \Delta S^\circ(\text{initi}) + \Delta S^\circ(\text{sym}) + \Delta S^\circ(\text{UG/AC}) \\ &\quad + \Delta S^\circ(\text{GC/CG}) + \Delta S^\circ(\text{CG/GC}) \\ &\quad + \Delta S^\circ(\text{GC/CG}) + \Delta S^\circ(\text{CA/GU}) \\ &= (-10.8) + (-1.4) + (-27.8) + (-34.9) + (-19.4) \\ &\quad + (-34.9) + (-17.8) \\ &= -157.0 \text{ cal K}^{-1} \text{ mol}^{-1},\end{aligned}$$

where ΔS°(initi) was an entropy change for the initiation of making the first base pair in the region, ΔS°(sym) was the correction entropy-factor for the self-complementary sequence, and ΔS°(UG/AC), ΔS°(GC/CG), ΔS°(CG/GC), ΔS°(GC/CG), and ΔS°(CA/GU) were propagation entropy-changes for making each subsequent base pair. Similarly, the enthalpy change

$$\Delta H^\circ = -57.4 \text{ kcal mol}^{-1}.$$

Therefore, the free-energy change at 37 °C, ΔG°_{37}, which was the stability energy of the UGCGCA duplex, was obtained as follows:

$$\begin{aligned}\Delta G^\circ_{37} &= \Delta H^\circ - T\Delta S^\circ \\ &= (-57.4) - (273 + 37) \times (-157.0 \times 10^{-3}) \\ &= -8.7 \text{ kcal mol}^{-1}.\end{aligned}$$

The value of ΔG°_{37} gives the equilibrium constant, K, of the transition of the single-stranded RNA to the double-helix 1.4×10^6 mol^{-1} dm^3 at 37 °C in Eq. 2:

$$K = \exp(-\Delta G^\circ_{37}/RT) = 1.4 \times 10^6 \text{ mol}^{-1} \text{ dm}^3. \quad (2)$$

Results and Discussion

HPLC Chromatograms of Substrate and Product at Each Reaction Time. The ligations as shown in Scheme 1 were performed under the standard conditions of an 1-hour to 20-hour incubation at 37 °C with an acceptor : donor ratio of 1 : 4. Figure 1 shows the HPLC chromatograms of the ligation mixture of AAUGCAU to pUp at several reaction times. As shown in Fig. 1, only two peaks which are due to the substrate AAUGCAU and the product AAUGCAUUp can be detected after a 40-min flow, although the peaks of pUp, ATP, ADP, and AMP are observed before a 30-min flow. The yields of the product were 51, 90, and 100% at 1, 4, and 8 h, respectively.

Ligation of Single-Stranded Acceptors to Pyrimidine and Purine Donors. The yields of the products in the reaction of single-stranded RNA acceptors to pyrimidine and purine donors are listed in Table 1 with the results of Romaniuk et al.[14] The melting temperatures of AAU and AUGCA which have the possibility of the formation of the self-complementary double-helix are calculated to be less than 0 and 29 °C, respectively, suggesting these oligomers are mainly single-stranded at 37 °C. In the case of pyrimidine donors in the ligation of AUG acceptor, the yield of the product for pCp donor is larger than that for pUp donor. The same tendency for the pyrimidine donors is observed in the reaction of AAU acceptor. In the reaction of the acceptors containing a different sequence and a different strand length (Entry Nos. 5—8 in Table 1), pCp donor also gives the larger yield than pUp.

In the case of purine donors in the ligation of AUGC acceptor, pAp donor gives the larger yield than pGp. The same tendency for the donors is detected in the reaction of AAU acceptor having a different sequence and a different strand length (Nos. 11 and 12 in Table 1).

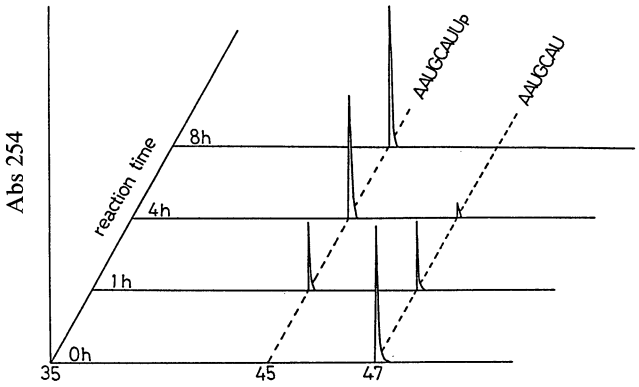


Fig. 1. HPLC chromatograms of AAUGCAU and AAUGCAUUp in the reaction mixture of the T4 RNA ligase reaction at 0, 1, 4, and 8 h.

Table 1. The Yield of the Product in the Reaction of Single-Stranded RNA Acceptors with pZp

Entry No.	Acceptor X···Y	Donor pZp	Enzyme concn U/ml	Time h	Yield %
1	AUG	C	30	1.5	31
2	AUG	U	50	2.5	12
3[a)]	AAU	C	175	1	98
4[a)]	AAU	U	175	1	75
5	AUGCA	C	30	1	55
6	AUGCA	U	30	1	18
7	AUGCG	C	50	1	62
8	AUGCG	U	50	1	3
9	AUGC	A	40	1	34
10	AUGC	G	50	1	9
11[a)]	AAU	A	175	1	63
12[a)]	AAU	G	175	1	17

a) Conditions: acceptor, 0.5 mM; donor, 1.0 mM; ATP, 3.3 mM; incubated at 37 °C in Ref. 14.

Ligation of Double-Stranded Acceptor to Pyrimidine and Purine Donors. Table 2 shows the stabilization energies at 37 °C, ΔG°_{37}, the melting temperatures, T_m, and the equilibrium constants, K, for the self-complementary double helices of UGCGCA, AUGCGCA, AUGCAU, and XAUGCAU (X=A, U, G, C). In Table 2, the calculated and observed T_m value for UGCGCA of 62.6 and 63.1 °C, respectively, suggests that the acceptor forms the self-complementary duplex at 37 °C which is the temperarure of the ligation in this work. The ΔG°_{37} value of −8.7 kcal mol^{-1} and the K value of 1.4×10^6 mol^{-1} dm^3 indicate that the oligomer exists almost as the duplex at 37 °C. In addition, the duplex of the oligomer may form an A-type double helix,[15] because, in Table 2, the T_m value calculated using A-type nearest-neighbor parameters[7,12] is consistent with the observed value. Therefore, all the results support the UGCGCA acceptor is not single-stranded but double-stranded at the reaction temperature.

The yields of the products in the reaction of double-stranded UGCGCA acceptor to pyrimidine and purine donors are listed in Table 3. In the case of pyrimidine donors, the yield of the product for pCp donor is larger than that for pUp donor. On the other hand, in the case of purine donors, pAp donor gives the larger yield than pGp. These are the same results observed in the case of the single-stranded RNA acceptor as shown in Table 1. Therefore, whether the acceptor is single-stranded or double-stranded does not affect the efficiency of the RNA ligation.

Ligation of Double-Stranded Acceptors Containing Dangling Ends to pZp Donors. In Table 2, the values of ΔG°_{37}, T_m, and K for AUGCGCA and XAUGCAU (X=A, C, G, U) show that these oligomers exist almost as the self-complementary duplexes at 37 °C as well as UGCGCA and AUGCAU. CD spectra of these oligomers support the above consideration: Figure 2 shows a typical CD spectrum of 10^{-3} mol dm^{-3} AAUGCAU in a 2×10^{-1} mol dm^{-3} Tris-HCl (pH 7.5), 2×10^{-2} mol dm^{-3} $MgCl_2$ buffer at 37 °C. The CD spectrum indicates a large positive peak at 278 nm and a small negative peak at 237 nm which are well-known to be due to a A-type RNA double helix.

In Table 2, the T_m value of AUGCGCA is 3.6 °C higher than that of UGCGCA. Also, in the case of the dangling ends on AUGCAU in the same table, the T_m values of XAUGCAU are about 4—6 °C higher than that of AUGCAU. The values of ΔG°_{37} in Table 2 show that the double helix having each dangling end is about 0.2 or 0.3 kcal mol^{-1} more stable than the core double helix of UGCGCA or AUGCAU. These results suggest the dangling nucleotide stabilizes the core double helix with its stacking interaction.[7]

In the ligation of purine donors to the AUGCGCA double-stranded acceptors having the A dangling end, the yield of the product for pAp donor is higher than that for pGp donor as shown in Entry Nos. 3 and 4 in Table 4. The result is the same in the cases of the single-stranded RNA acceptors and the double-stranded UGCGCA acceptor. In contrast, in the case of pyrimidine donors, pUp donor gives the yield of 12% higher than pCp, even under the lower enzyme unit of 25 U/ml, in Nos. 1 and 2 in Table 4, which is the opposite tendency as observed in the above cases, that is, the cases of the single-stranded and double-stranded accep-

Table 2. The Stabilization Energies, the Melting Temperatures, and the Equilibrium Constants of the Self-Complementary RNA Double Helixes

Oligomer	$-\Delta G^\circ_{37}$	T_m[a]	T_m[b]	K
	kcal mol^{-1}	°C	°C	mol^{-1} dm^3
UGCGCA	8.7	63.1	62.6	1.37×10^6
AUGCGCA	9.0		66.2	2.20×10^6
AUGCAU	5.1	42.9	40.6	4.12×10^3
AAUGCAU	5.4		46.3	6.71×10^3
CAUGCAU	5.4		45.3	6.71×10^3
GAUGCAU	5.4		46.4	6.71×10^3
UAUGCAU	5.3		44.6	5.70×10^3

a) The calculated melting temperature for the oligomer of 10^{-3} mol dm^{-3}. b) The measured melting temperature for the oligomer of 10^{-3} mol dm^{-3}.

Table 3. The Yield of the Product in the Reaction of Double-Stranded UGCGCA Acceptors with pZp

Entry No.	Acceptor X···Y	Donor pZp	Enzyme concn	Time	Yield
			U/ml	h	%
1	UGCGCA / ACGCGU	C	50	1	70
2	UGCGCA / ACGCGU	U	50	1	22
3	UGCGCA / ACGCGU	A	50	1	42
4	UGCGCA / ACGCGU	G	50	1	13

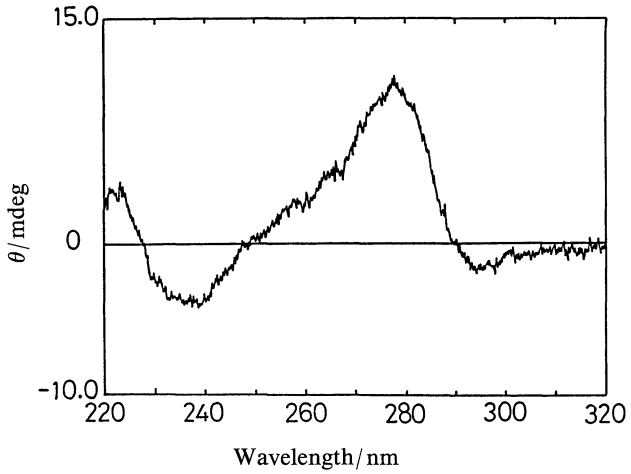


Fig. 2. CD spectrum of 10^{-3} mol dm^{-3} AAUGCAU in a buffer containing 2×10^{-2} mol dm^{-3} $MgCl_2$ and 2×10^{-1} mol dm^{-3} Tris-HCl (pH 7.5) at 37 °C.

Table 4. The Yield of the Product in the Reaction of Self-Complementary Double-Stranded AUGCGCA and CAUGCAU Acceptors Having Dangling Ends with pZp

Entry No.	Acceptor X···Y	Donor pZp	Enzyme concn U/ml	Time h	Yield %
1	AUGCGCA / ACGCGUA	C	50	1	69
2	AUGCGCA / ACGCGUA	U	25	1	81
3	AUGCGCA / ACGCGUA	A	50	1	49
4	AUGCGCA / ACGCGUA	G	90	1	17
5	CAUGCAU / UACGUAC	C	50	1	84
6	CAUGCAU / UACGUAC	U	50	1	42
7	CAUGCAU / UACGUAC	A	50	1	73
8	CAUGCAU / UACGUAC	G	50	1	100

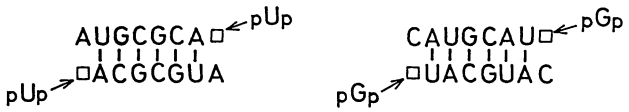


Fig. 3. The schematic illustrations of pUp and pGp donors approaching the dangling **A** and **C** nucleotide on AUGCGCA and CAUGCAU self-complementary duplexes, respectively.

tors. Even in comparison with pyrimidine donors, the pUp donor gives the highest yield of the product for 1 h. The result leads to the consideration that the pUp donors can approach most easily the dangling **A** nucleotide with a base-pair interaction[15] between the U and A nucleotides as shown in Fig. 3.

In the reaction of the double-stranded CAUGCAU acceptor having the **C** dangling end, pyrimidine donors show the same tendency observed in the single-stranded and double-stranded acceptors having no dangling end, but purine donors indicate the opposite tendency, that is, pGp gives the yield of the product 27% higher than pAp (Nos. 5—8 in Table 4). The yield of the product for pGp is the highest value for all the donors. The result supports the consideration that a base-pair interaction between the **G** and **C** nucleotides in Fig. 3 has a large effect on the ligation. Therefore, the base-pair interaction in the case of the **A** and **C** dangling ends accelerates the RNA ligation.

It follows that the base-pair formation in the reaction of the acceptors having different dangling ends to the same donor should accelerate the reaction rate more than the mismatch interactions such as GA, GU, and GG interactions.[16] In order to examine the above idea, the ligation of the pGp donor to the double stranded XAUGCAU (X=A, C, G, U) acceptors were

Table 5. The Yield of the Product in the Reaction of Double-Stranded RNA Acceptors Having Dangling Ends with pGp

Entry No.	Acceptor X···Y	Donor pZp	Enzyme concn U/ml	Time h	Yield %
1	AAUGCAU / UACGUAA	G	50	1	50
2	CAUGCAU / UACGUAC	G	50	1	100
3	GAUGCAU / UACGUAG	G	50	1	37
4	UAUGCAU / UACGUAU	G	50	1	41

studied. In Table 5, the 100% yield of the product for CAUGCAU is the largest value in comparison with the 50, 37, and 41% yield for AAUGCAU, GAUGCAU, and UAUGCAU, respectively. The result suggests the base-pair interaction between the dangling X nucleotide on the core double helix and the pZp donor, in general, accelarate the rate of the RNA ligation by its making the donor approaching easily the ligation site of the acceptor. Therefore, the study in this paper suggests that the interaction between the substrates such as the base-pair interaction between the unpaired terminal nucleotide on the double helix of the RNA acceptor and the donor as well as the interaction of the substrates with the enzyme,[17] plays a very important role in the RNA ligation with T4 RNA ligase.

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