CYCLIZATION AND DIMERIZATION OF HEXANUCLEOTIDES CONTAINING GUANINE AND CYTOSINE WITH WATER-SOLUBLE CARBODIIMIDE

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(Received 10 September 2001 Accepted 9 November 2001)

(Abstract)
The condensation reaction of hexanucleotides 5'-pGGGCCrC, 5'-pGCGCGrC, and 5'-pGCCCGrG was investigated in the presence of water-soluble carbodiimide (WSC) and imidazole at 0 °C. The efficiency of the condensation of hexanucleotides with WSC was investigated to know whether these hexanucleotides act as a partial template to promote the elongation reaction. The condensation reaction mainly yielded cyclic-hexanucleotides (30 - 60 %) and small amount of 12-mers (< 10 %), which opposed our expectation. The yield of cyclization was in the order 5'-pGGGCCrC ≅ 5'-pGCGCGrC > 5'-pGCCCGrG and that of dimerization was in the order 5'-pGGGCCrC ≅ 5'-pGCGCGrC < 5'-pGCCCGrG. The trend to cause efficient cyclization could be an obstacle at an early stage of chemical evolution of RNA.

(Keyword)
Oligonucleotide, RNA, Phosphodiester bond, Cyclicnucleotide, Template-directed reaction, RNA world, Ligation

Introduction

Ribonucleic acids (RNA) preserve both information and catalytic functions, so the RNA or RNA-like molecules played important roles under primitive earth environments [1]. If the RNA world hypothesis is correct then RNA could have been accumulated under prebiotic conditions. The condensation reactions of nucleotides with and without using activated monomer under primitive earth conditions have been evaluated as primitive polymerase models of nucleotide oligomers. Examples include the condensation of activated 5'-GMP on a poly(C) template [2], the condensation of activated 5'-pApA on a $(pU)_{10}$ template [3], and the spontaneous condensation of the activated nucleotide monomer in the presence of metal ions [4] and clay mineral catalysts [5]. The phosphodiester bond formation using condensation agents have also been investigated. Minimal replicating system was succeeded using short oligonucleotides in the presence of water-soluble carbodiimide [6]. Moreover, the condensation of hexanucleotide on a poly(U) template [7] and the condensation of trinucleotide in the presence of trivaline catalyst have been evaluated [8].

Based on these prebiotic syntheses of oligonucleotides, it would be deduced a following scenario of the chemical evolution of RNA. First, at the first stage towards the RNA world, oligonucleotides spontaneously formed in the presence of catalysts such as metal ions and clay minerals but without template nucleotides, and then the reproduction of ribonucleotides occurred through pathways such as the template-directed reaction. Thus, during the spontaneous formation in the presence of metal ion and clay catalysts, four types of bases could have been incorporated into oligonucleotide sequences. This may be not expedient for the replication of oligonucleotides by the template-directed reaction, since the efficiency of the template-directed reaction is not high for the formation of pyrimidine oligonucleotides on a purine polynucleotide template [2]. Thus, it is important to search plausible elongation pathways other than the conventional template-directed reaction [3,5]. In this study, as a connecting pathway between the spontaneous formation and the replication of RNA, we supposed that short oligonucleotides could elongate through a route as shown in Fig. 1a, in which the hexanucleotide acts as a partial template of itself.

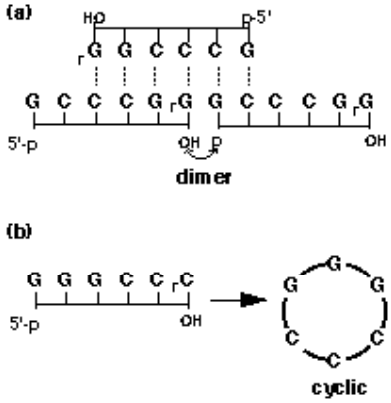

Figure 1. Models of the condensation of hexanucleotides. (a) double-tracking template-directed formation of 12-mer from 6-mer. (b) cylization of a hexanucleotide.

To evaluate this model, the condensation of hexanucleotides with WSC was investigated using three types of hexanucleotides. Conclusively, the condensation reaction caused mainly cyclization (Fig. 1b), but the efficiency of the elongation from oligo-6-1, and -6-2 was very low and that from oligo-6-3 was slightly high.

Experimental

Hexanucleotides 5'-pGGGCCrC (oligo-6-1), 5'-pGCGCGrC (oligo-6-2), and 5'-pGCCCGrG (oligo-6-3) were purchased from GENSET (France) as HPLC purified grade. Ribonuclease T_2 (RNase T_2) and alkaline phosphatase (APH) were purchased from SIGMA. All other reagents used were of analytical grade. T_2

The condensation reactions were performed in an aqueous solution containing NaCl, $MgCl_2$, imidazole, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (WSC), and 0.05 – 0.1 mM hexanucleotide at 0 °C. The solution containing WSC was freshly prepared for each reaction. The pH of the sample solutions was adjusted with 0.1 M NaOH or 0.1 M HCl solution. The mixture was allowed to stand for 7 days and aliquots were taken over 7 days, which was immediately quenched in liquid nitrogen. The samples were analysed by HPLC on a DNA-NPR anion exchange column from TOSOH Co., Tokyo, Japan using a gradient of 0 - 1.2 M NaCl at pH 11 with 0.02 M 2-amino-2-hydroxymethyl-1,3-propanediol (tris). The kinetic analyses of the reaction curves were performed using the computer program SIMFIT [6].

The structures of oligonucleotides were determined by selective enzymatic hydrolyses with RNase T_2 and APH [9]. RNase T_2 hydrolysis was carried out in 0.1 mL reaction mixture for 1 to 18 h at 37 °C at pH 4.5 using 0.05 - 0.5 unit of enzyme, and APH hydrolysis was performed in 0.1 mL for 30 min at 37 °C with 1 unit of enzyme.

Results and Discussion

Stability of WSC

It was confirmed by HPLC analysis that both WSC and imidazole are essential to proceed the condensation reaction, since oligo-6 in the absence of WSC or imidazole did not form any products within 7 days. UV spectra of the solution containing NaCl, $MgCl_2$, imidazole, and WSC was monitored at 200 - 300 nm for 7 days, but the change in absorbance was not detected (Fig. 2). Thus, it is regarded that the activity of WSC remains constant for 7 days at 0 °C during the condensation experiments of hexanucleotides. The maximum wavelength of the absorption spectra of WSC in the absence of imidazole (213 nm) was sifted to 232 nm in the presence of imidazole (Fig. 2). Based on these facts, it is assumed that phosphorimidazolide of hexanucleotide formed in the presence of WSC and imidazole, and then proceeded the condensation reactions.

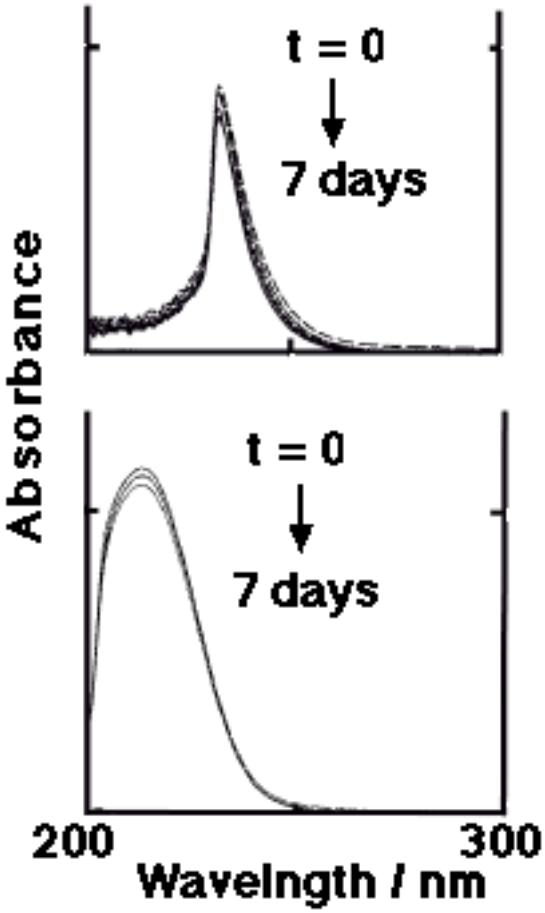

Figure 2. Absorption spectral change of WSC under the condensation reaction conditions but in the absence of hexanucleotides.

[WSC] = 0.2 M, [NaCl] = 0.2 M, [$MgCl_2$] = 0.075 M, pH = 8.0, 0 °C. Top: 0.1 M imidazole, bottom: no imidazole.

Characterisation of the products

Fig. 1a shows a model that we expected for elongation of hexanucleotides [10]. This model is considered as a variation of the template-directed reaction and may be called as the double-track elongation reaction. The 3'-terminal of hexanucleotides is RNA and other sequences are DNA with 5'-phosphate, since it is known that phosphodiester bond of ribose forms more efficiently than that of deoxyribose by the condensation reaction in the presence of WSC under several conditions [2,6,8]. In addition, reasons that the hexanucleotides containing guanine and cytosine were selected in this study are follows. First, Watson-Crick base pair formation between guanine and cytosine is stronger than that between adenine and uracil [11]. Further, it has been repeatedly described that early genes were constructed from guanine and cytosine rich nucleic acids rather than adenine and uracil rich [12].

The reactions of oligo-6-1, -6-2, and -6-3 were monitored by HPLC and two main products were found as peak-1 and peak-2 of HPLC (Fig. 3).

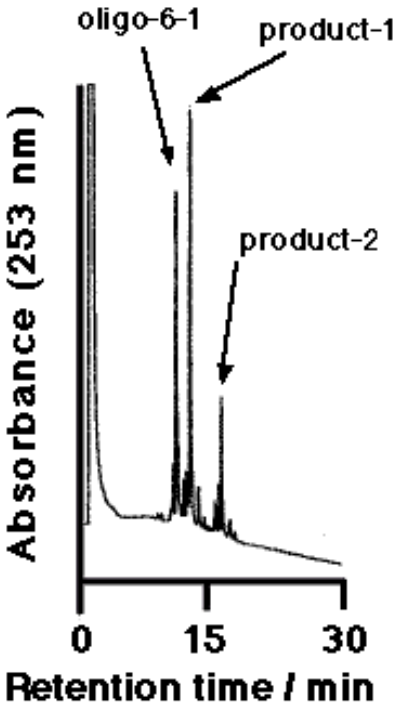

Figure 3. HPLC charts of the products from the condensation of pGGGCCrC (oligo-6-1).
Anion-exchange column (TOSOH Co., DNA-NPR), HPLC buffer : tris : 0.02 M, NaCl gradient, pH = 11.

The yield of peak-1 was higher than that of peak-2 for three types hexanucleotides tested in this study. Since the retention time of peak-2 of oligo-6-1 was consistent with that of authentic 12-mer (5'-pGGGCCCGGGCCC), peak-2 was assigned as a dimerized product of oligo-6-1. For further characterisation of peak-1, enzymatic hydrolysis with RNase T_2 and APH was performed. Peak-1 disappeared by hydrolysis with RNase T_2 so the peak-1 involves 3',5'-linked ribose phosphodiester bond. Besides, peak-1 was not degraded by APH hydrolysis. Based on these analyses, peak-1 is assigned as cyclic hexanucleotide (cyclic-6-mer) and peak-2 is assigned as dimerized products (12-mer) of hexanucleotides. Thus, the condensation reaction with WSC mainly proceeds cyclization through the pathway as shown in Fig. 1b for oligo-6-1 and -2. This is coincidence with previous studies, in which the cyclic nucleotides were easily formed under the prebiotic synthesis of oligonucleotides [4, 9].

The time course of the reactions of oligo-6-1, -2, and -3 was followed for 24 h at 0 °C (Fig. 4). Cyclic-6-mers were the main products for oligo-6 and a small amount of 12-mer was observed for oligo-6-1 and -2. The yield of 12-mer from oligo-6-3 was somewhat high compared to oligo-6-1 and -2. The ratio of the extents of elongation to cyclization in average was 0.045 for oligo-6-1, 0.017 for oligo-6-2,

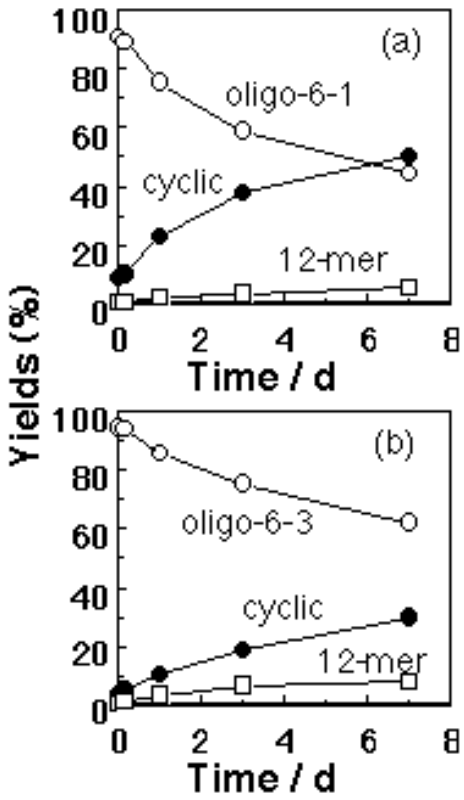

Figure 4. Reaction profiles for the condensation of hexanucleotides.
[NaCl] = 0.2 M, [$MgCl_2$]= 0.075 M, [Imidazole] = 0.1 M, pH = 8.0, 0 °C, [oligo-6-1 or -3] = 1 x 10^{-4} M, [WSC] = 0.2 M.
(a) 5'-pGGGCCrC, (b) 5'-pGCCCGrG.

and 0.15 for oligo-6-3. The relatively high yield of 12-mer from oligo-6-3 may be due to following reasons. First, for the case of oligo-6-1, two molecules of oligo-6-1 are capable to form double helix since self-complementary, where the double helix involves 6 base pairs and does not act as a template, and the same type of double helix can be formed for the case of oligo-6-2. However, the possible double helix involves only 4 base pairs for oligo-6-3 (Fig. 1a). Second, the linkage being produced by the condensation of oligo-6-1 or -2 is the ligation between G and C, but that for oligo-6-3 is that between G and G. In previous studies, it was elucidated that the formation of associate of activated monomer and elongating oligomer is important for the elongation of oligonucleotides [9,13,14]. The association between G and G for oligo-6-3 may be more effective for the formation of 12-mer than that between G and C for oligo-6-1 and –2 (Fig. 2).

Rate of the formation of cyclization and elongation of hexanucleotides

First, it was observed that salt compositions did not affect much the condensation reaction. Besides, the influence of the WSC and oligo-6 concentrations for the condensation reaction was detected. Both the yields of cyclic-6-mer and linear 12-mer

increased in the presence of twice of WSC, while were not much changed those in the presence of twice of oligo-6. This is due to that WSC was added in large excess of hexanucleotide, where the condensation rate is proportional to the WSC concentration (Table 1, condition 3,4).

Table 1. The rate constants for the formation of cyclichexanucleotides and dimerization of hexamers.

	nulceotides	k_{cyc} / s^{-1}	k_{dim} / s^{-1} M^{-1}
1	6-1	$(4.6\pm0.1)\times10^{-7}$	ND
	6-2	$(6.3\pm0.1)\times10^{-7}$	ND
	6-3	$(2.7\pm0.1)\times10^{-7}$	$(1.2\pm0.2)\times10^{-3}$
2	6-1	$(6.4\pm0.1)\times10^{-7}$	ND
	6-2	$(2.9\pm0.1)\times10^{-7}$	ND
	6-3	$(9.9\pm1.0)\times10^{-8}$	$(6.9\pm1.3)\times10^{-4}$
3	6-1	$(8.9\pm0.3)\times10^{-7}$	$(6.0\pm2.0)\times10^{-4}$
	6-2	$(6.8\pm0.1)\times10^{-7}$	$(1.8\pm1.1)\times10^{-4}$
	6-3	$(2.8\pm0.1)\times10^{-7}$	$(8.2\pm1.0)\times10^{-4}$
4	6-1	$(1.6\pm0.1)\times10^{-6}$	$(1.2\pm0.3)\times10^{-3}$
	6-2	$(1.6\pm0.1)\times10^{-6}$	$(6.7\pm3.9)\times10^{-4}$
	6-3	$(6.9\pm0.1)\times10^{-7}$	$(1.6\pm0.2)\times10^{-3}$

Reaction Conditions, 1: [NaCl] = 0.1 M, [$MgCl_2$] = 0.1 M, [imidazole] = 0.1 M, [WSC] = 0.1 M, pH = 7.5; 2: [NaCl] = 1.0 M, [$MgCl_2$] = 0.2 M, [imidazole] = 0.1 M, [WSC] = 0.1 M, pH = 8.0; 3: [NaCl] = 0.2 M, [$MgCl_2$] = 0.075 M, [imidazole] = 0.1 M, [WSC] = 0.1 M, pH = 8.0; 4: [NaCl] = 0.2 M, [$MgCl_2$] = 0.075 M, [imidazole] = 0.1 M, [WSC] = 0.2 M, pH = 8.0. Hexanucleotides, 6-1: 5'-pGGGCCrC, 6-2: 5'-pGCGCGrC, 6-3: 5'-pGCCCGrG.

The reaction curves of oligo-6 (Fig. 4) were fitted using a following reaction model and the apparent rate constants of cyclization (k_{cyc}) and dimerization (k_{dim}) were determined by SIMFIT (Table 1).

oligo-6 → cyclic-6-mer (1)

oligo-6 + oligo-6 → oligo-12 (2a)

oligo-6 + oligo-6 + oligo-6 → oligo-12 + oligo-6 (2b)

According to the model shown in Fig. 1a, the dimerzation of oligo-6 should obey pseudo-third order kinetics. However, dimerization was detected even in the reactions of oligo-6-1 and –2, which seem to be unlikely to follow pseudo-third order rate. Thus, both models expressed by equations 2a and 2b were tested, where pseudo-second-order rate model gave somewhat better fitting. This fact may indicate that dimerization of oligo-6s follows pseudo-second-order rate rather than pseudo-third-order rate. However, since the yield of dimerization is not very high in these reactions, detail analysis would be important to determine whether the dimerization is template dependent or independent. The values of k_{dim} decreased in the order oligo-6-3 > oligo-6-1 > oligo-6-2. Besides, the magnitude of k_{cyc} was comparable for oligo-6-1 and -2, but that of oligo-6-3 was somewhat smaller than that of oligo-6-1 and -2. The difference between the ligation within G and G for oligo-6-3 and that within G and C for oligo-6-1 and –2 may be important. These facts suggest that the low efficiency of cyclization of oligo-6-3 is also important factor to determine the yield of dimerization.

On the Chemical Evolution of RNA

The effective formation of cyclic-6-mer was unexpected and this is coincidence with some previous studies, in which rapid cyclization reactions were observed during the formation of oligonucleotides from activated nucleotide without template [4,9]. The cyclization of short peptides also occurs during the formation of oligopeptides under prebiotic conditions [15]. Thus, the cyclization of short oligomers during the formation of biopolymers could have been inevitable under prebiotic conditions since both amino acids and nucleic acids have two functional groups. In other word, some chemical pathways would be necessary to prevent from the cyclization of short oligomolecules. Besides, the dimerization of oligo-6 follows pseudo-second or –third-order kinetics so that the apparent rate of the dimerization is dependent on the concentration of hexanucleotide, while cyclization follows pseudo-first-order kinetics. Thus, dimerization of biomolecules could be promoted with increasing the concentrations of monomer unit.

The yield of oligonucleotides for the spontaneous formation and the template-directed reaction is dependent on the type of bases, length of oligonucleotide, and whether 3',5'- or 2',5'-linkage. For example, the spontaneous formations of oligonucleotides in the presence of metal ion or clay catalysts are not a base specific reaction [4,5,9], but the condensation from mixtures of activated mononucleotides on the clay catalysts is dependent on the sequence of oligonucleotide and whether 2',5'- or 3',5'-linkage [16]. Besides, the template-directed reaction using an activated mononucleotide is highly efficient only for the case of the formation of oligo(G) on a poly(C) template [2,17]. Recently, it was elucidated that the efficiency of the template-directed reaction can be enhanced by stabilising double-helical association of elongating unit and template [3]. In these studies on the spontaneous formation and the template-directed formation of RNA, the knowledge on the sequence dependence was limited. The present study showed that the

elongation and cyclization are possible to be sequence dependent and this is useful to estimate what sequence of oligonucleotides have been effectively formed under primitive earth conditions.

Conclusions

In this study, it was found that the condensation of hexanucleotides oligo-6-1, -2, -3 in the presence of WSC and imidazole yielded mainly cyclic-hexanucleotides and a small amount of dimerized product of hexanucleotides. The fact that the efficiency of cyclization is much higher than that of dimerization suggests that the cyclization of oligonucleotides could be a problem for the accumulation of RNA. Moreover, it was observed that both the cyclization and dimerization are sequence dependent even though oligo-6-1, -2, and –3 have the same G and C composition.

Acknowledgement

This research was supported by the Sumitomo Foundation 1998 and the Mazda Foundation's Research Grant 2000. We thank Professor T. Nakahara in Department of Applied Chemistry for the use of HPLC in Osaka Prefecture University. Professor G. von Kiedrowski in Ruhr-Universitaet Bochum generously provided SIMFIT.

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