Kinetics and Activation Parameter Analysis for the Prebiotic Oligocytidylate Formation on Na^+-Montmorillonite at 0−100 °C

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The kinetic analysis of the temperature dependence of the formation of oligocytidylate (oligo(C)) from the 5′-monophosphorimidazolide moiety of cytidine (ImpC) in the presence of Na^+-montmorillonite (Na^+-Mont) catalyst has been carried out at 0−100 °C. The rate constants for the formation of oligo(C), hydrolysis of ImpC with and without Na^+-Mont and degradation of oligo(C) were determined. The apparent activation parameters were 30.8 ± 3.9 kJ mol^{-1} (*E*a), 28.3 ± 4.0 kJ mol^{-1} ($\Delta H^{\ddagger}$), and −231 ± 13 J mol^{-1} K^{-1} ($\Delta S^{\ddagger}$) for the formation of the 2-mer; 45.6 ± 2.9 kJ mol^{-1} (*E*a), 43.0 ± 3.0 kJ mol^{-1} ($\Delta H^{\ddagger}$), −164 ± 10 J mol^{-1} K^{-1} ($\Delta S^{\ddagger}$) for the 3-mer; and 45.2 ± 0.6 kJ mol^{-1} (*E*a), 42.7 ± 0.7 kJ mol^{-1} ($\Delta H^{\ddagger}$), −159 ± 2 J mol^{-1} K^{-1} ($\Delta S^{\ddagger}$) for the 4-mer in the presence of Na^+-Mont. An increasing trend for the rate constants for the formation of oligo(C) in the order 2-mer ≪ 3-mer <4-mer was observed at high temperatures, which is consistent with that observed at low temperatures. These analyses implied for the first time that the associate formation between an activated nucleotide monomer and an elongating oligonucleotide prior to the phosphodiester bond formation during the elongation of an oligonucleotide on a clay surface would be based on the interaction between the two reactants at the phosphoester and/or ribose moieties rather than at the nucleotide bases. The hydrolysis rate of ImpC at 25−100 °C was 5.3−10.6 times greater in the presence of Na^+-Mont than in its absence. Although the degradation of oligo(C) in the presence of Na^+-Mont was slower than the formation of the 3-mer and longer oligo(C) on Na^+-Mont, its yield decreased with temperature. This is mainly because the ratios of the rate constant of the 2-mer formation to those of ImpC hydrolysis and the 3-mer and 4-mer formation decrease with an increase in temperature, which is attributed to the enthalpy and entropy changes for the formation of the 2-mer. This trend resembles the case of the template-directed formation of oligo(G) on a poly(C) template but is different from the Pb^{2+}-ion-catalyzed oligo(C) formation. According to the kinetics and activation parameter analyses regarding the clay reaction and other prebiotic polymerase models, the possible pathways for the oligonucleotide formation are discussed and compared.

Introduction

The discovery of catalytic RNA molecules has suggested that RNA or RNA-like molecules could have played a central role in the emergence of life on the primitive earth.[1–3] If this hypothesis is correct, the RNA molecules should be accumulated under simulated prebiotic conditions. This assumption has been experimentally verified by a number of successful studies on the condensation reactions of activated nucleotides to form RNA oligonucleotides in the presence of polynucleotide templates (TD reaction),[4–6] metal ion catalysts,[7–9] or clay mineral catalysts (CL reaction).[10–14] In these prebiotic reactions, the activated nucleotide monomers are considered as prebiotic compounds, which can be formed from inorganic phosphate, nucleotide monomer, and imidazole.[15,16] Further investigation has suggested that the activated monomer could have formed in fairly high concentrations.[17] Kinetic investigations have provided an insight into these reaction mechanisms.[11,18–20] In addition, it has been elucidated that long oligonucleotides could have been formed from monomeric nucleotides without the involvement of an enzyme catalyst.[4–6,21–23] Furthermore, oligocytidylate (oligo(C)) formed by the CL reaction can be preserved as a prebiotic template for the formation of oligo(G) by the TD reaction.[13,14] However, the RNA world hypothesis has some drawbacks despite being supported by other empirical evidence.[2,3,24] In particular, the hypothesis that life originated under hydrothermal vent environments (the hydrothermal origin of life hypothesis) appears to be inconsistent with the RNA world hypothesis.[25–30]

The hydrothermal origin of life hypothesis was proposed on the basis of the observation of thermophilic organisms[31–33] and the phylogenetic analysis of present organisms. The last common ancestor (LCA) has been considered by many to have been a thermophilic organism;[34,35] however, this is occasionally disputed.[36–41] It would be important to focus on some technical aspects that need to be solved.[42] In addition, the temperature of the primitive ocean in which life originated remains speculative.[43–45] Although the LCA might not be hyperthermophilic, it should be at least thermophilic.[46]

A few investigations concerning the degradation of RNA and its precursors have been conducted from the standpoint of the hydrothermal origin of life.[25,27,29,30,47–50] According to the empirical data regarding the stability of RNA molecules, it is considered that the RNA molecules are too labile under redox-constrained hydrothermal conditions,[51,52] although some minerals appear to protect nucleotides and their precursors from degradations.[53,54] On the other hand, while the prebiotic formation of RNA was rarely investigated at high temperatures,[55] we

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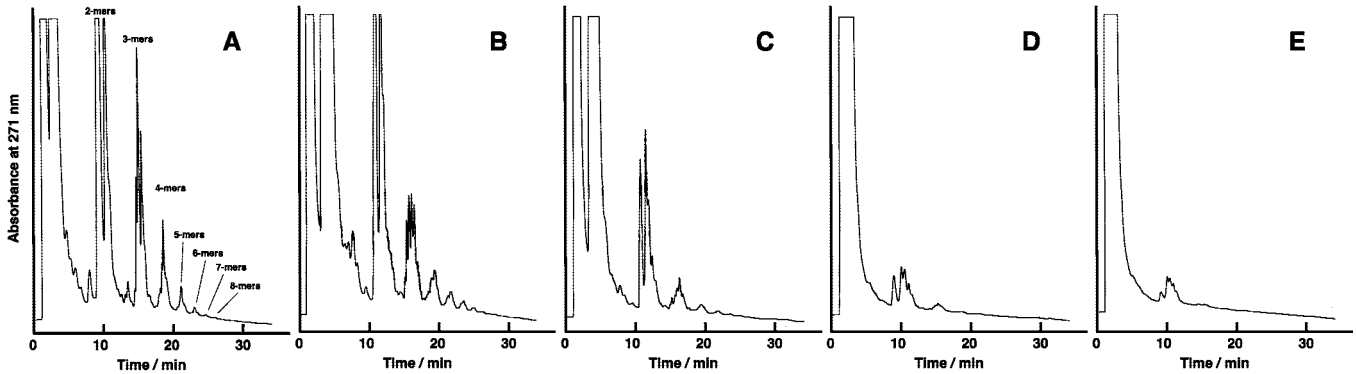

Figure 1. HPLC charts for the oligo(C) formation in the presence of Na^+-Mont. Reaction conditions: [ImpC] = 0.015 M, [NaCl] = 0.2 M, [$MgCl_2$] = 0.075 M, [HEPES] = 0.1 M, pH = 8.0. Aqueous phase, 1 mL; Na^+-Mont, 50 mg. (A) 0 °C, 480 h; (B) 5 °C, 336 h; (C) 50 °C, 24 h; (D) 75 °C, 100 min; (E) 100 °C, 30 min. Samples were analyzed by AE-HPLC.

have recently accumulated kinetic data on the temperature dependence of prebiotic RNA polymerase model reactions, that is, the TD reaction, cyclization reaction of hexanucleotides, and Pb^{2+}-ion-catalyzed oligonucleotide formation (PB reaction).[56–58] These investigations not only showed that the phosphodiester bond becomes labile at high temperatures but also suggested that its prebiotic formation could be faster than its degradation at high temperatures.[56–58] Naturally, this estimation does not give a direct proof of the accumulation of the RNA molecules could under the primitive hydrothermal conditions. Nevertheless, it would be theoretically true that the accumulation of the RNA molecules may be kinetically controlled in an open system by both the formation and decomposition rates of RNA, even at high temperatures.[51,59] In particular, it has been elucidated that mineral catalysts could have played important roles in the spontaneous formation of long oligonucleotides from monomeric nucleotides.[14,21,22] Thus, for the RNA world hypothesis and hydrothermal origin of life hypothesis to be compatible, it is important to accumulate kinetic data regarding the temperature dependence of the prebiotic simulation reactions of the RNA molecules to validate the rate of the prebiotic formation of RNA at high temperatures. Furthermore, the analysis of the activation parameters would provide a valuable insight into the reaction mechanism of the prebiotic formation of RNA. In the present study, the kinetic analysis of the formation of oligo(C) in the presence of a Na^+-Mont catalyst has been carried out at 0−100 °C. The kinetic analysis and activation parameters of the CL reaction were evaluated by comparing them with those of the previous investigations concerning the TD and PB reactions. The reaction of oligo(C) formation is important. Since kinetic analyses for the CL reactions using different activated nucleotide monomers have been carried out, the scope and comparison of different CL reactions would provide insights into the reaction mechanism.[11,18–20,60] A plausible pathway of the formation of cytosine has been proposed so that a reasonable amount of cytosine could have been present on the early Earth.[61] Furthermore, the oligo(C) formation could have played important roles since oligo(C) formed by the CL reaction can be preserved as a prebiotic template for the formation of oligoguanylates (oligo(G)).[12,13]

Experimental Section

Materials and Equipment. The 5′-monophosphorimidazolide moiety of cytidine (ImpC) was prepared by the techniques mentioned in previous studies.[6,62] Montmorillonite VolClay was gifted by Professor J. P. Ferris (Rensselaer Polytechnic Institute) from American Colloid Company, Arlighton Heights, IL. Na^+-Mont was prepared from VolClay following the techniques mentioned in previous studies.[10,11] Ribonuclease A (RNaseA) (Bovine Pancrease, XII-A type or I-AS type) was obtained from SIGMA. All the other reagents used were of analytical grade. High-performance liquid chromatography (HPLC) was carried out on an LC10A HPLC system (Shimadzu, Japan) with a DNA-NPR anion-exchange column from Tosoh Co., Japan using a gradient of 0.3−1.5 M NaCl at pH 9 with 0.02 M 2-amino-2-hydroxymethyl-1,3-propanediol (Tris) buffer and an ODS-2 column from GL Science Co., Japan using a gradient of 0.005 M NaH_2PO_4 in water at pH 3.5 mixed with 0.01 M NaH_2PO_4 in 40% CH_3OH at pH 4.0.[58]

Oligomerization of ImpC in the Presence of Na^+-Mont at Elevated Temperatures. The reaction solutions were prepared by dissolving 6 mg of ImpC in a 1 mL solution containing 0.015 M ImpC, 0.2 M NaCl, 0.075 M $MgCl_2$, and 0.1 M 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) at pH 8.0. A 0.015 M solution of ImpC was added to 50 mg of Na^+-Mont and mixed by vortexing in a 1.5 mL plastic vial (RNase free) that can withstand temperatures up to 120 °C. The pH of the mixture was adjusted to 8.0 using a small amount of NaOH solution. The vial was placed in a cartridge temperature controller at 0−100 °C. The sample solution was withdrawn at regular intervals over 480 h at 0 °C, 336 h at 25 °C, 30 h at 50 °C, 2 h at 75 °C, and 80 min at 100 °C and then immediately quenched with liquid nitrogen. Control reactions without Na^+-Mont were carried out at 25−100 °C. The samples were analyzed by both anion-exchange HPLC (AE-HPLC) and reversed-phase HPLC (RP-HPLC). The calculation of the rate constants was performed using a kinetic program SIMFIT.[11,63]

Degradation of Oligo(C) at Elevated Temperatures. A standard solution containing abiotic oligo(C) was prepared from the oligo(C) formation catalyzed by Na^+-Mont at 25 °C for 7 days or longer. Running solutions containing the standard oligo(C) were incubated at 60−90 °C, and aliquot samples were withdrawn at regular intervals. Oligo(C) with 3′,5′-linkage was prepared by the partial hydrolysis of an authentic poly(C) sample. This was also used for the inspection of the hydrolytic stability in the presence of Na^+-Mont. The samples were analyzed by AE-HPLC. The rate constants of the degradation of oligo(C) were determined on the basis of the previous method.[56,58,59]

Enzymatic Degradation of Oligo(C). A 30−50 μL solution containing 20 unit/mL of alkaline phosphatase (APH) was added to a 30−50 μL sample solution, and the mixture was incubated for 2 h at 37 °C. A 5−30 μL solution containing RNaseA

TABLE 1: Yields (%) of Oligo(C) from ImpC in the Presence of Na^+-Mont at Different Temperatures[a]

T/°C	time/h	ImpC	$^{5'}$pC	2-mer	3-mer	4-mer	5-mer	6-mer	7-mer	C$^{5'}$ppC
0	480	31.8	43.6	10.5	2.6	1.0	0.3	0.1	<0.1	9.9
25	48	33.4	51.6	6.3	1.6	0.6	0.3	0.1	<0.1	6.0
50	4	28.0	67.0	1.6	0.3	0.1	<0.1	0.0	0.0	3.0
75	1.67	32.2	62.8	0.7	0.1	<0.1	0.0	0.0	0.0	4.3
100	0.5	22.5	68.8	0.3	0.0	0.0	0.0	0.0	0.0	8.3

[a] Reaction conditions are the same as shown in Fig. 1. The percentages are based on the concentrations of products.

TABLE 2: Apparent Rate Constants for the Hydrolysis of ImpC and the Formation of Oligo(C)[a]

	T/°C				
	0	25	50	75	100
$k_{hy,ImpC}/s^{-1}$	$(6.27 \pm 0.05) \times 10^{-7}$	$(5.98 \pm 0.05) \times 10^{-6}$	$(7.09 \pm 0.04) \times 10^{-5}$	$(2.69 \pm 0.03) \times 10^{-4}$	$(7.59 \pm 0.04) \times 10^{-4}$
$k_2/M^{-1}\,s^{-1}$	$(1.53 \pm 0.02) \times 10^{-5}$	$(6.94 \pm 0.11) \times 10^{-5}$	$(1.80 \pm 0.05) \times 10^{-4}$	$(2.74 \pm 0.32) \times 10^{-4}$	$(2.97 \pm 0.17) \times 10^{-4}$
$k_3/M^{-1}\,s^{-1}$	$(7.69 \pm 0.21) \times 10^{-5}$	$(6.00 \pm 0.12) \times 10^{-4}$	$(2.04 \pm 0.04) \times 10^{-3}$	$(6.02 \pm 0.19) \times 10^{-3}$	$(7.74 \pm 5.10) \times 10^{-3}$
$k_4/M^{-1}\,s^{-1}$	$(1.85 \pm 0.11) \times 10^{-4}$	$(1.01 \pm 0.02) \times 10^{-3}$	$(4.00 \pm 0.08) \times 10^{-3}$	$(1.20 \pm 0.04) \times 10^{-2}$ [c]	
$k_{py}/M^{-1}\,s^{-1}$	$(3.95 \pm 0.06) \times 10^{-5}$	$(1.30 \pm 0.03) \times 10^{-4}$	$(6.08 \pm 0.08) \times 10^{-4}$	$(4.14 \pm 0.18) \times 10^{-3}$	$(1.54 \pm 0.02) \times 10^{-2}$
$k_{hy,ImpC}/s^{-1}$ [b]		$(9.74 \pm 0.95) \times 10^{-7}$	$(6.69 \pm 0.21) \times 10^{-6}$	$(3.44 \pm 0.25) \times 10^{-5}$	$(1.43 \pm 0.01) \times 10^{-4}$

[a] Error levels were determined from the standard deviation calculated by SIMFIT. [b] Control reactions in the absence of Na^+-Mont. [c] The magnitude of k_4 was defined as 1.99 times greater than that of k_3.

(3000−300 000 unit/mL) was added to a 5−30-μL sample solution, and the mixture was incubated for 18 h at 37 °C.

Results

Formation of Oligo(C) in the Presence of a Clay Catalyst. The formation of oligo(C) was monitored using AE-HPLC, and the disappearance of ImpC and the formation of cytidine 5′-monophosphate ($^{5'}$pC) and P_1,P_2-dicytidine 5′,5′-pyrophosphate (C$^{5'}$ppC) were analyzed using RP-HPLC.[56,58] The representative chromatograms are shown in Figure 1. The extents of ImpC, $^{5'}$pC, oligo(C), and C$^{5'}$ppC are summarized in Table 1, and the reaction curves are shown in Figure 2. The extent of oligo(C) decreases with an increase in temperature, where oligo(C)s up to 7-mer were observed at 0−25 °C, while oligo(C)s up to 3-mer were detected at 100 °C. Enzymatic treatments with APH and RNaseA showed that the extent of 2′,5′-linked oligo(C) was 79% for the oligo(C) products at 25 °C. This is consistent with the results of the previous investigations.[13] The yield of oligo(C) decreased both with its length and temperature. These trends are similar to those observed in the case of the TD and PB reactions.[11,18–20,56,58] No oligo(C) was detected in the control reaction in the absence of Na^+-Mont during the hydrolysis of ImpC to $^{5'}$pC. The reaction curves obtained in the absence of Na^+-Mont are shown in Figure 3. Naturally, the reaction rates increased, while the yield of oligo(C) decreased with an increase in the temperature.

Degradation of Oligo(C) at High Temperatures. Oligo(C)s up to 3-mer were observed at 100 °C, while their yields were low. It is necessary to investigate the degradation rate of oligo(C) formed by the CL reaction to clarify whether its degradation is slower than its formation in the presence of Na^+-Mont at high temperatures or not. The rate of the oligo(C) degradation during the CL reaction was difficult to observe at temperatures over 50 °C since the yields of oligo(C) were low. Thus, the degradation of oligo(C), which was prepared in advance by the CL reaction at 25 °C, was monitored in the presence of Na^+-Mont at 60−90 °C for the comparison of its formation and degradation rates. The degradation rates were examined on the basis of the disappearance of the phosphodiester bond. The mole concentration of the total number of phosphodiester bonds (C_{bond}) was calculated from the concentration of oligo(C) (C_{oligo}) using eq 1[56,58,59]

$$C_{bond} = C_{oligo} \Sigma A_i((i\text{-}1)/i) \quad (1)$$

where A_i indicates the ratio of the oligo(C) of length i. Here, the time course of the disappearance of C_{bond} was monitored, where it is reasonable to assume that the rate of cleavage of the phosphodiester bond is independent of the values of i.[56,58,59] In the present case, the experiment indicates that the degradation of oligo(C)s, mainly those including 2′,5′-phosphodiester-linked isomers, was investigated in the presence of Na^+-Mont. The reaction curves are shown in Figure 4. It was confirmed that the rate of disappearance of the oligo(C)-phosphodiester bond increases with temperature.

Rate Constants of the Clay Catalyzed Formation of Oligo(C). The reaction rate constants in the CL reaction system were expressed by the following reaction model,[11,20]

$$\text{ImpC} \xrightarrow{k_{hy,ImpC}} {}^{5'}\text{pC} \quad (2\text{-}1)$$

$$\text{ImpC} + {}^{5'}\text{pC} \xrightarrow{k_{py}} \text{C}^{5'}\text{ppC} \quad (2\text{-}2)$$

$$\text{ImpC} + {}^{5'}\text{pC} \xrightarrow{k_2} (\text{pC})_2 \quad (2\text{-}3)$$

$$\text{ImpC} + (\text{pC})_2 \xrightarrow{k_3} (\text{pC})_3 \quad (2\text{-}4)$$

$$\text{ImpC} + (\text{pC})_3 \xrightarrow{k_4} (\text{pC})_4 \quad (2\text{-}5)$$

$$\text{ImpC} + (\text{pC})_{n-1} \xrightarrow{k_n} (\text{pC})_n \quad (2\text{-}6)$$

$$\text{oligo(C)} \xrightarrow{k_{hy,oligo}} \text{degradation} \quad (2\text{-}7)$$

where $(pC)_2$, $(pC)_3$, $(pC)_{n-1}$, and $(pC)_n$ indicate the 2-mer, 3-mer, $(n-1)$-mer, and n-mer oligo(C)s. The rate constants, k_2, k_3, k_4, and k_n correspond to the formation of the 2-mer, 3-mer, 4-mer, and n-mer, respectively, where k_4 is defined as a constant value for 4-mer and longer oligo(C)s ($k_n = k_4$). The consecutive

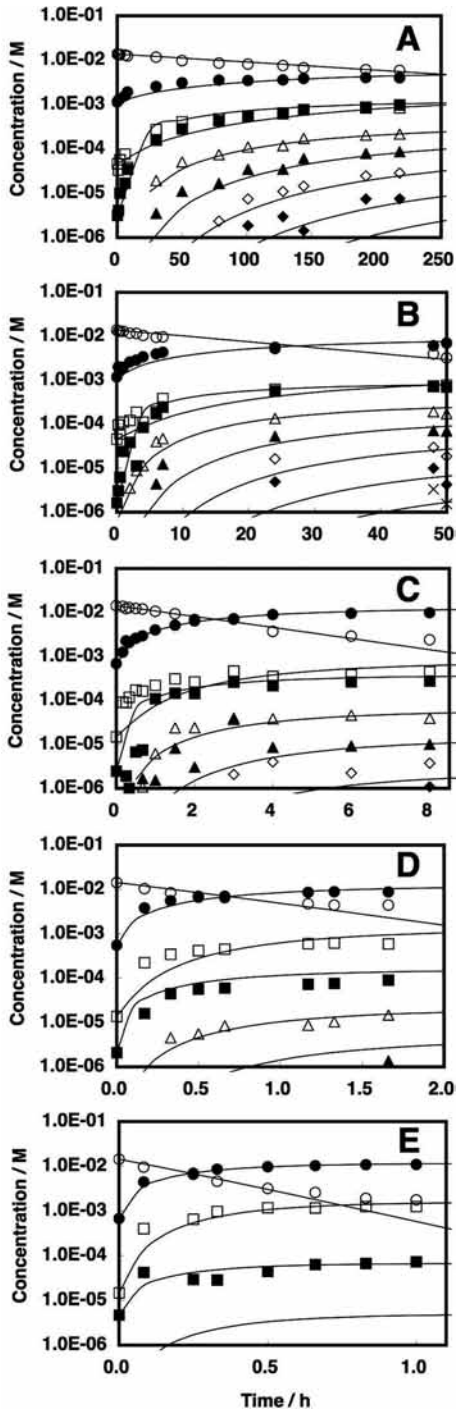

Figure 2. Reaction curves for the oligo(C) formation in the presence of Na^+-Mont. Reaction conditions are the same as shown in Figure 1. The lines drawn through the experimental points were fit by SIMFIT. (A) 0 °C; (B) 25 °C; (C) 50 °C; (D) 75 °C; (E) 100 °C. Open circles, ImpC; closed circles, $^{5'}$pC; open squares, C$^{5'}$ppC; closed squares, 2-mer; open triangles, 3-mer; closed triangles, 4-mer; open diamonds 5-mer; closed diamonds, 6-mer; crosses, 7-mer.

elongation model given in eqs 2-1–2-6 has been extensively evaluated for the CL reaction using different types and concentrations of the activated nucleotide monomers[11,20,60] and also for the TD reactions.[18,19,56] For the case of the CL reaction using ImpC, no cyclic oligo(C) was detected in the dimer and longer oligo(C) fractions in the HPLC analyses although a large amount of cyclic 3-mers were detected for the cases of 5′-monophosphorimidazolide of inosine (ImpI) and 5′-monophosphorimidazolide of uridine (ImpU).[20] Thus, it is obvious

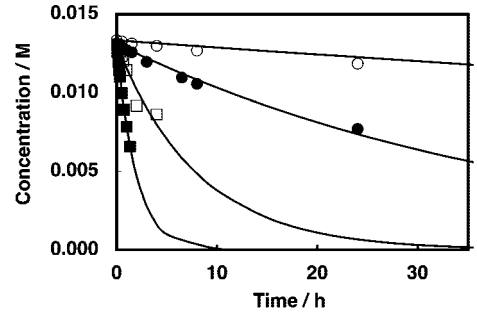

Figure 3. Reaction curves for the disappearance of ImpC in the absence of Na^+-Mont. Reaction conditions are the same as shown in Figure 1. The lines drawn through the experimental points were fit by SIMFIT. Temperatures: open circles, 25 °C; closed circles, 50 °C; open squares, 75 °C; closed squares, 100 °C.

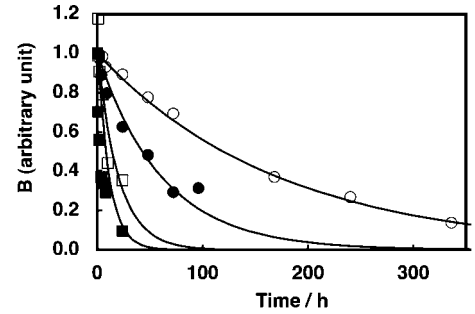

Figure 4. Reaction curves for the disappearance of oligo(C) in the presence of Na^+-Mont. Oligo(C) was prepared by the CL reaction at 25 °C and then performed for degradation experiment at 60−90 °C. C_{bond} indicates the concentration of phosphodiester bond. The lines drawn through the experimental points were fit by SIMFIT. Temperatures: open circles, 60 °C; closed circles, 70 °C; open squares, 80 °C; closed squares, 90 °C.

that the reaction of ImpC obeys the standard reaction model shown in eqs 2-1–2-6. The rate constants for the formation of C$^{5'}$ppC and ImpC hydrolysis were defined as k_{py} and $k_{hy,ImpC}$, respectively. Besides, the hydrolytic degradation of oligo(C) proceeds during the CL reaction at high temperatures. The rate constant $k_{hy,oligo}$ was calculated from the reaction curves for the hydrolytic degradation of oligo(C). According to the previous study, the binding of ImpC was comparable to that of ImpU at 25 °C.[13,64] While the bindings of ImpC and the oligo(C)s on Na^+-Mont in the present system can be considered on the basis of the previous kinetic model,[11] the adsorption of ImpC and oligo(C)s was not determined on Na^+-Mont at high temperatures since the determination of the binding constants is experimentally difficult and the binding of these molecules is very low at high temperatures. Hence, we have decided to evaluate the apparent rate constants in the presence of Na^+-Mont since the former are sufficiently useful for the kinetic and mechanistic analysis of the CL reactions, as shown in the previous study.[20] The reaction curves shown in Figure 2 were fitted using SIMFIT, and the rate constants are summarized in Table 2. It was possible to obtain convergent rate constants k_2 and k_3 at 0−100 °C, while the value of k_3 at 100 °C involves a significant error. Over 100 data points were used for the determination of 5 rate constants (k_{hy}, k_{py}, k_2, k_3, and k_4) for each kinetic analysis at different temperatures 0 − 50 °C, and ca. 50 data points were used for 4 or 3 rate constants at 75−100 °C. According to previous kinetic studies using SIMFIT,[20,60,63,65,66] the number of data points is regarded as sufficiently large on the basis of the ratio of the number of data points to the number of unknown rate constants. The rate constants k_4 at 75 °C were calculated by

TABLE 3: Degradation Rate Constants of Oligo(C) Formed by the Clay Catalytic Reaction of ImpC[a]

	T/°C			
	60	70	80	90
$k_{hy,oligo}/s^{-1}$	$(1.61 \pm 0.03) \times 10^{-6}$	$(4.52 \pm 0.24) \times 10^{-6}$	$(1.36 \pm 0.24) \times 10^{-5}$	$(2.43 \pm 0.33) \times 10^{-5}$

[a] Error levels were determined from the standard deviation of the pseudofirst-order rate plots.

assuming that the magnitude of k_4 was 1.99 times greater than that of k_3 to reduce the number of unknown variables. The error levels of HPLC readings increased with decreasing the extent of reaction products, where error levels were 0.15% for the analysis of a 0.01 M product, 1% for 1 mM, 3.5% for 0.3 mM, and 7.5% for 0.1 mM. Error levels of the rate constants are indicated as the values of standard deviation evaluated by SIMFIT. The error levels of the rate constants increase with increasing the length of oligo(C) is consistent with the trend that the error levels of HPLC readings increase with the length of oligo(C). Since the number of data points used for the determination of the rate constants would be sufficiently large, the error levels of the rate constants became comparable to or better than those of HPLC readings.

In addition, the rate constants of the hydrolysis of ImpC in the absence of Na^+-Mont were determined. On an average, the rate constant for the ImpC hydrolysis ($k_{hy,ImpC}$) in the presence of Na^+-Mont was 7.5 times greater than that in the absence of Na^+-Mont at 25−100 °C, while no oligo(C) was detected in the absence of Na^+-Mont. This fact suggests that Na^+-Mont accelerates the hydrolysis of ImpC, which is consistent with the results of previous studies.[11,20]

The hydrolytic degradation of oligo(C), which was prepared by the CL reaction, was determined at 60−90 °C in the presence of Na^+-Mont. The pseudofirst-order rate constants were determined from the initial rates (Table 3). The error levels were determined from the standard deviation of the first-order-rate plots. The magnitude of the rate constant for the oligo(C) hydrolysis at 80 °C is fairly similar to that for the hydrolytic degradation of C2′pC at 80 °C,[50] although the conditions used in these experiments are very different. This fact indicates that Na^+-Mont may not possess any notable catalytic or inhibitory activity for the hydrolytic degradation of oligo(C). Indeed, the hydrolytic stability of 3′,5′-linked oligo(C) in the presence of Na^+-Mont was qualitatively monitored at 60−90 °C. The stability of the 3′,5′-linked oligo(C) was fairly low, where most of the oligo(C) disappeared within 1 day, even at 60 °C.

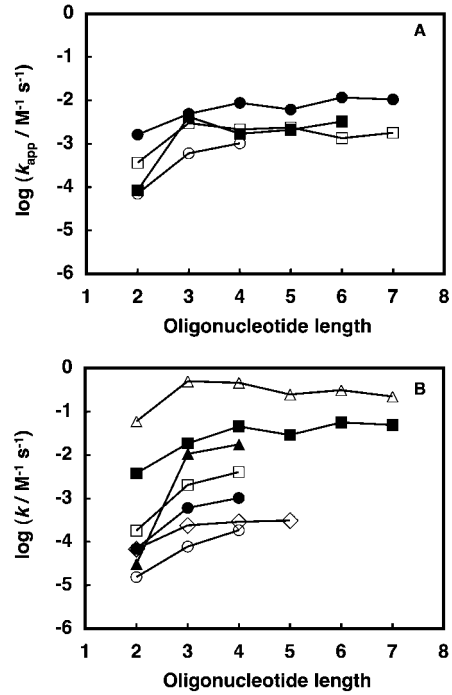

Figure 5. Relationships between logarithmic values of the rate constants for the oligonuleotides formation and the oligonucleotide length. (A) Apparent rate constants (k_{app}) in the presence of Na^+-Mont vs oligonuleotide length; open circles (ImpC, present study), closed circles (ImpA),[11,20] open squares (ImpU),[20] closed squares (ImpI).[20] The rate constants are apparent rate constants, of which the contributions of the clay phase are not corrected. (B) Rate constants in different prebiotic model reactions for oligonucleoide formation. CL reaction using ImpC in the present study: open circles, 0 °C; closed circles, 25 °C; open squares, 50 °C; closed squares, CL reaction using ImpA (25 °C); [11] open triangles, CL reaction using ImpU (25 °C);[20] closed triangles, TD reaction using 2-MeImpG;[20] open diamonds, PB reactions using ImpC.[58] The rate constants for the cases of CL reactions using ImpA or ImpU are the values on the clay phase.

Discussion

Kinetics. The apparent rate constants for the elongation of oligo(C) in the presence of Na^+-Mont can be compared with those for different activated nucleotide monomers in the previous studies since all the reaction conditions are the same, except for the type of nucleotide base of the activated nucleotide monomers. The magnitudes of k_2, k_3, and k_4 from the ImpC formation are fairly smaller than those for the cases of 5′-monophosphorimidazolide of adenosine (ImpA), 5′-monophosphorimidazolide of inosine (ImpI), and 5′-monophosphorimidazolide of uridine (ImpU), where the magnitude of k_2 decreases in the order ImpA > ImpU > ImpI, ImpC, and that of k_4 decreases in the order ImpA > ImpU, ImpI, ImpC (Figure 5A). Nevertheless, the present study supports the fact that in general, the apparent rate constants for the formation of oligonucleotides are not notably dependent on the type of the activated nucleotide monomer.

In the present case, an increasing trend was observed for the magnitude of the rate constant for the oligo(C) formation in the order $k_2 \ll k_3 < k_4$ (Table 2, Figure 5B) at 0−50 °C; furthermore, the magnitude of k_3 was greater than that of k_2 at 75 °C. This trend was the basis for the reaction mechanism proposed in the previous study.[11] Furthermore, the above trend is similar to that observed in the TD reaction,[18,19,56] but is fairly clearer than the case of the PB reaction (Figure 5B).[58] To describe the differences in the above trend between the CL and TD reactions and the PB reaction, the formation of oligonucleotides in different prebiotic model reactions is expressed in a general form given in eqs 3-1 and 3-2

$$*\mathrm{pNu} + \mathrm{oligo(Nu)}_i \overset{K_{ass}}{\leftrightarrow} *\mathrm{pNu} \cdot \cdot \cdot \mathrm{oligo(Nu)} \quad (3\text{-}1)$$

$$*\mathrm{pNu} \cdot \cdot \cdot \mathrm{oligo(Nu)}_i \overset{k_{phos}}{\rightarrow} \mathrm{oligo(Nu)}_{i+1} \quad (3\text{-}2)$$

$$k_{elong} = K_{ass} k_{phos} \quad (3\text{-}3)$$

where *pNu and oligo(Nu)$_i$ indicate a monomeric activated nucleotide and an elongating oligonucleotide of length i, respectively; *pNu•••oligo(Nu)$_i$ is the associate of *pNu and

oligo(Nu)$_i$; and oligo(Nu)$_{i+1}$ is an oligonucleotide of length $i + 1$. K_{ass} is the equilibrium constant of the associate formation; k_{phos} is the rate constant of the phosphodiester bond formation from the associate; and k_{elong} is the overall rate constant. According to this model, the overall rate constants in a prebiotic model reaction would increase with the association between two activated nucleotide monomers or that between an activated monomer and an elongating oligonucleotide. The details of the importance of associate formation prior to the formation of the phosphodiester bond were verified in the case of the TD reaction.[18,19] The fact that the difference in the magnitudes between k_2 and k_4 increases with temperature indicates that the associate formation shown in eq 3-1 is important for the CL reaction as well as the TD reaction on a polynucleotide template. However, this appears to be less effective for the PB reaction, which was performed under the previously investigated conditions.[58]

The above mentioned trend ($k_2 \ll k_3 < k_4$) is more clearly observed for the cases of ImpC and ImpA as compared to the cases of ImpU and ImpI. This is probably due to the following reason. In the case of ImpI or ImpU, it was shown that the 2-mer fraction detected by AE-HPLC contained a large amount of the cyclic 3-mer since the present AE-HPLC method separates the components based on the number of negative charges on each oligonucleotide.[20] Thus, the magnitudes of k_2 in these cases involved the contribution from the formation of the cyclic 3-mers. It was verified that the cyclic 3-mers are less involved for the present case of oligo(C) formation from ImpC.[13] Thus, the fact that there is a difference between k_2 and k_4 among the CL reactions using different activated nucleotide monomers supports the previously proposed reaction mechanism (Figure 5A).[11,20] Although the rate constants for the oligo(C) formation increased with temperature, the difference between k_2 and k_4 (or k_3) also becomes larger. The pH values of the aqueous phase, which was prepared at pH 8.0 at 25 °C, would change slightly to 7.7 (50 °C), 7.5 (°C), 7.2 (100 °C),[67] while the temperature dependence of the clay hydration is unknown. Naturally, the temperature dependence would be more important for predicting the reaction behavior. Thus, the fact that the rate of formation of the 2-mer becomes relatively small is a reason that the efficiency of the oligo(C) formation decreases with temperature, which has been evaluated by a mathematical simulation for 5′-monophosphorimidazolide of guanosine (ImpG) on a poly(C) template.[56]

The trend ($k_2 \ll k_3 < k_4$) was not dependent on the type of the activated nucleotide monomers. In addition, the total efficiency of the yield of oligonucleotides was not notably influenced by the type of activated nucleotide monomer, in contrast to the case of the TD reactions. In other words, the importance of the associate formation prior to the phosphodiester bond formation may not be dependent on the type of the activated nucleotide monomers. This was unexpected since it appears to be inconsistent with the fact that the associate formation between a nucleotide monomer and an elongating oligomer would be enhanced by increasing the π-π stacking between the nucleotide bases. For the case of the TD reaction, this assumption is supported by the fact that this reaction proceeds efficiently from ImpG on a poly(C) template but does not proceed at all from ImpC on a poly(G) template. However, in the case of the CL reaction, the efficiency of the associate formation between an activated nucleotide monomer and an elongating olignucleotide would not be directly correlated with the base stacking between the activated nucleotide monomers. Conclusively, in the CL reaction, this fact indicates that the phosphoester moieties and/or ribose moieties would be more important than the base stacking for the associate formation between two activated monomers or that between an activated monomer and an elongating oligonucleotide.

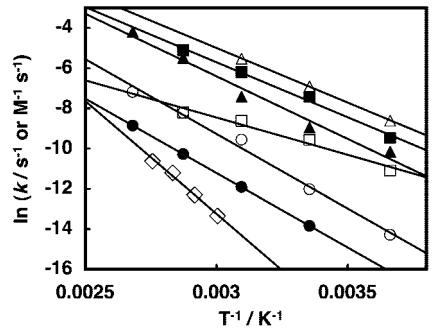

Figure 6. Arrhenius plots for rate constants regarding the oligo(C) formation in the presence of Na^+-Mont. Lines: open circles, $k_{hy,ImpC}$ in the presence of Na^+-Mont; open squares, k_2; closed squares, k_3; open triangles, k_4; closed triangles, k_{py}; open diamonds, $k_{hy,oligo}$; these rate constants are obtained in the presence of Na^+-Mont; closed circles, $k_{hy,ImpC}$ in the absence of Na^+-Mont.

On the other hand, the adsorption of the activated nucleotide monomers is dependent on the type of the base, where the adsorption decreases in the order of ImpG ~ ImpA > ImpC ~ ImpU.[11–13,20,60,64,68] The previous investigations showed that the strength of adsorption of the activated nucleotide monomers is predominantly determined by the hydrophobicity of the nucleotide base.[11,20] According to the previous studies,[20,64] the net adsorption of an activated nucleotide monomer could involve an effective adsorption and a noneffective adsorption for the oligonucleotide formation. Thus, an increase in the hydrophobicity of the activated nucleotide monomers and elongating oligonucleotides would not directly enhance the probability of the effective adsorption of these molecules onto the active site of Na^+-Mont.[20] This fact is supported by the CL reaction of ImpC. An increasing trend for the rate constants was observed in the order $k_2 \ll k_3 < k_4$ at high temperatures, but the ratio of k_2 to k_4 decreased with temperature. This fact indicates that the associate formation for the 2-mer formation becomes distinctly weak when compared with that for the 3-mer and 4-mer formations. These facts support the existence of active sites on Na^+-Mont, which catalyze the formation of the phosphodiester bond between an activated nucleotide monomer and an elongating oligonucleotide.

Activation Parameter Analysis. On the basis of the temperature dependence of the rate constants (Figure 6), the magnitudes of the apparent activation energy (Ea), enthalpy change ($\Delta H^{\ddagger}$), and entropy change ($\Delta S^{\ddagger}$) were calculated (Table 4). The error levels were determined from the standard deviation of the Arrhenius plots (Figure 6) and Eyring plots for logarithmic values of the rate constants versus T^{-1}. According to the generalized reaction model for the prebiotic formation of oligonucleotides shown in eqs 3-1 and 3-2, the formation of oligo(C) on Na^+-Mont can be divided into the following processes: (1) adsorption of ImpC and elongating oligo(C) on Na^+-Mont, (2) associate formation of ImpC and oligo(C) on Na^+-Mont, (3) formation of phosphodiester bond from the associate on Na^+-Mont, and (4) desorption of the elongating oligo(C). Thus, the activation parameters in the CL reaction involve these processes, whereas processes (1) and (2) are involved in the pathway shown in eq 3-1. The magnitudes of Ea and $\Delta H^{\ddagger}$ for k_2 are notably smaller than those for k_{py}, k_3, and k_4. In addition, the values of $\Delta S^{\ddagger}$ for k_2 are smaller than those for k_3 and k_4. This fact indicates that the formation of

TABLE 4: Apparent Activation Parameters Regarding the Reactions of the ImpC System[a]

corresponding rate constants	Ea/kJ mol^{-1}	$\Delta H^{\ddagger}$/kJ mol^{-1}	$\Delta S^{\ddagger}$/J mol^{-1} K^{-1}
$k_{hy,ImpC}$	61.7 ± 3.4	59.0 ± 3.4	−146 ± 11
k_2	30.8 ± 4.4	28.3 ± 4.0	−231 ± 13
k_3	45.6 ± 2.9	43.0 ± 3.0	−164 ± 10
k_4	45.2 ± 0.6	42.7 ± 0.7	−159 ± 2
k_{py}	51.7 ± 4.4	49.1 ± 4.3	−152 ± 14
$k_{hy,ImpC}$ (control without clay)	61.5 ± 0.1	58.8 ± 0.1	−163 ± 1
$k_{hy,oligo}$	93.3 ± 7.0	90.4 ± 7.0	−85 ± 20

[a] Error levels were determined from the standard deviation of Arrhenius plots or Eyring plots.

2-mer is advantageous for $\Delta H^{\ddagger}$, but disadvantageous for $\Delta S^{\ddagger}$ as compared with the formation of 3-mer and 4-mer. Besides, the magnitudes of Ea, $\Delta H^{\ddagger}$, and $\Delta S^{\ddagger}$ for the formation of C$^{5'}$ppC are comparable to those for the formation of 3-mer and 4-mer. These facts suggest that the probability of a suitable configuration for forming the phosphodiester bond via the associate between two ImpC monomers and/or between ImpC and $^{5'}$pC to form the 2-mer would be very limited as compared to that of the formation of a longer oligo(C). On the contrary, such a rigid conformation at the transition state would not be necessary for the formation of C$^{5'}$ppC.

Unexpectedly, the magnitudes of Ea and $\Delta H^{\ddagger}$ for $k_{hy,ImpC}$ in the presence of Na^+-Mont are almost the same as those in the absence of Na^+-Mont. This fact indicates that the enhancement of the hydrolytic degradation of ImpC on Na^+-Mont is mainly due to the entropy change, indicating configuration that is favorable for the hydrolytic degradation of phosphorimidazolide on Na^+-Mont; OH^- ions may more readily attack the phosphorimidazolide on the Na^+-Mont surface than in the bulk solution.

On the other hand, a comparison of the kinetic parameters among the CL, TD, and PB reactions would be useful to verify the characteristics of the CL reaction. Naturally, the TD and PB reactions do not involve the adsorption and desorption of the reactants on the clay surface. However, the TD reaction involves the association and dissociation processes of the activated monomer and elongating oligonucleotide for a polynucleotide template. Similarly, the PB reaction involves the association and dissociation of these molecules through Pb^{2+} ion catalysts. Thus, a careful comparison of the activation parameters of the present system with those of the other reactions is necessary. In general, the trend of the activation parameters observed in the present CL reaction resembles that in the TD reaction rather than that in the PB reaction. The magnitudes of Ea and $\Delta H^{\ddagger}$ for the oligonucleotide formation in the CL reaction are smaller than those in the TD and PB reactions.[56–58] This fact indicates that the CL reaction is more advantageous for the phospho-transformation process than the TD and PB reactions. In particular, the values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ for the 2-mer formation in the CL reaction are smaller than those in the TD and PB reactions. This would be the possible reason for the low efficiency of the oligonucleotide formation at high temperatures since the 2-mer formation would not be effective at high temperatures. The magnitudes of both $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ increased in the order $k_2 < k_3 < k_4$ for both the CL and TD reactions (Figure 7). This fact reflects that the strain in the conformation prior to the formation of the phosphodiester bond decreases in the order 2-mer > 3-mer > 4-mer. This is also consistent with the fact that the extents of C$^{5'}$ppC for the CL reaction or G$^{5'}$ppG for the TD reaction increased with temperature. Furthermore, the activation parameters were recalculated to be 86.7 ± 12.3 kJ mol^{-1} (Ea), 83.9 ± 12.3 kJ mol^{-1} ($\Delta H^{\ddagger}$), and −53 ± 37 J mol^{-1} K^{-1} ($\Delta S^{\ddagger}$) on the basis of the rate constants for the formation of G$^{5'}$ppG in the TD reaction.[56] Thus, it was confirmed that the magnitudes of the activation parameters of k_{py} in the CL reaction are also smaller than those in the TD reaction.

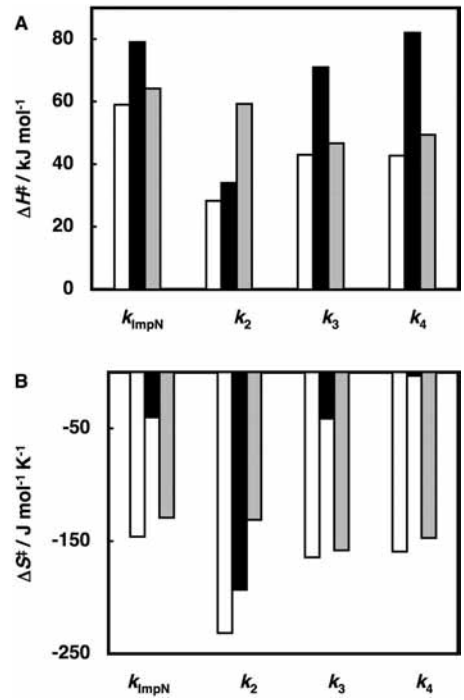

Figure 7. Comparison of the values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ for the hydrolysis of the activated nucleotide monomers and the oligonucleotides formation. (A) $\Delta H^{\ddagger}$; (B) $\Delta S^{\ddagger}$; open columns, the present CL reaction using ImpC (present data); closed columns, TD reaction using ImpG;[56,57] halftone columns, PB reaction using ImpC.[58]

The fact that the yields of higher oligonucleotides in the CL reaction are smaller than those in the TD reaction, but greater than those in the PB reaction would be attributed to the nature of the entropy change for the formation of the 2-mer in the CL reaction. By comparing the CL and TD reactions, we can state that the alignment of an elongating oligonucleotide and an activated nucleotide monomer on the clay surface or template polynucleotide may be the possible reason for the entropy changes accompanying the CL reaction being smaller than those accompanying the TD reaction. The alignment of the reactants on the template molecule in the TD reaction would be maintained by hydrogen bonding and nucleotide base stacking within the Watson−Crick-type double helix. However, the alignment of the reactants in the CL reaction can be maintained solely by the adsorption of these molecules on the clay surface and the associate formation between the reactants. The hydrogen bonding of the reactants on the template in the TD reaction is apparently more directional than the adsorption of the reactants on the clay surface in the CL reaction.[18,19] The negative charge density of the clay surface does not appear to be not as high as that of the polynucleotide template if the negatively charged surface acts as a template for aligning the activated nucleotide monomers and enhancing the association between two monomers.[69,70] The charge density would not be so affected at 0−100 °C since it is basically determined by the composition of the clay. In addition, the present study showed that the base stacking would not be very important for the associate formation between the reactants in the CL reaction (Figure 8). Thus, it would be

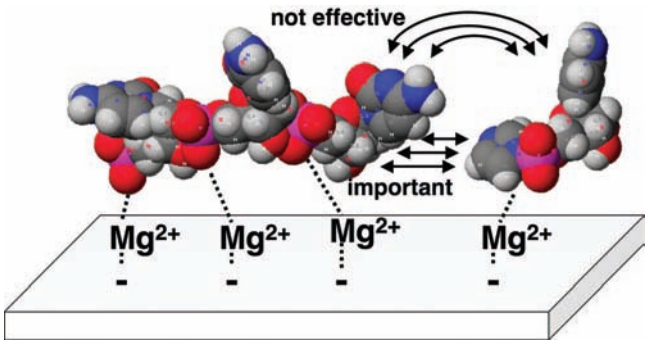

Figure 8. Proposed model for clogation of oligonucleotide by reaction with an activated nucleotide monomer on the clay surface.

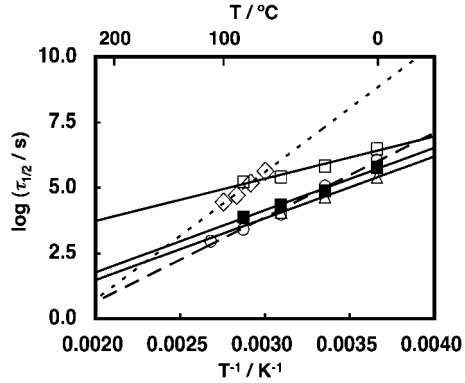

Figure 9. Logarithmic values of apparent rate constants regarding the oligo(C) formation in the presence of Na^+-Mont. Apparent rate constants for k_2, k_3, and k_4 were calculated at 0.015 M ImpC. Open circles, $k_{hy,ImpC}$ in the presence of Na^+-Mont; open squares, k_2; closed squares, k_3; open triangles, k_4; open diamonds, $k_{hy,oligo}$.

reasonable to state that the formation of the 2-mer in the CL reaction is disadvantageous from the viewpoint of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ as compared to that in the TD. For the phosphodiester bond formation on the clay surface, the interaction between two activated nucleotide monomers on the phosphoimidazolide side would be more important than that on the nucleotide base side. This is consistent with the fact that the apparent efficiencies of the CL reactions are not very strongly dependent on the type of the activated nucleotide bases as compared with the case of the TD reactions.

Reaction Behaviors of Oligo(C) Formation at Different Temperatures. Although the dimensions of $k_{hy,oligo}$ (s^{-1}) and $k_{hy,ImpC}$ (s^{-1}) are the same, they are different from those of k_2, k_3, and k_4 (M^{-1} s^{-1}). Thus, a direct comparison among $k_{hy,oligo}$, k_2, k_3, and k_4 would be meaningless. However, the kinetic analysis shows that the degradation rate of oligo(C) in the CL reaction is apparently slower than its formation rate at high temperatures based on their reaction behaviors. This trend is the same as the that observed in the TD and PB reactions.[56,58] Here, the apparent half-lives of the present CL reaction system at 0.015 M ImpC were calculated, and their logarithmic values ($\tau_{1/2}$) were plotted as a function of T^{-1} (Figure 9). The apparent half-lives for the 3-mer and 4-mer formation are shorter than that for the degradation of ImpC, although the half-life for the 2-mer formation is comparative to that for the latter. This trend is somewhat different from the case of the TD and PB reactions, where the apparent half-lives for the hydrolysis of the activated nucleotide monomers are considerably longer than those for the 3-mer and 4-mer formations. Naturally, this relationship is dependent on the concentration of ImpC since the ImpC degradation and oligo(C) degradation have first order-rate constants. Thus, with an increase in the ImpC concentration the rate of formation of oligucleotides becomes significantly greater than that of the degradation of oligo(C). An extrapolation of the present kinetic analyses of the prebiotic formation of oligonucleotides indicates that the formation rates of higher oligonucleotides could be greater than their degradation rates at fairly high temperatures, similar to those mentioned in the previous studies.[56,58] Although this perspective may be true, such high concentrations of the activated nucleotide monomer are unlikely to be formed under the primitive earth conditions. Thus, this fact does not directly indicate the possibility of the oligonucleotide formation under hydrothermal environments. Nevertheless, the kinetic analyses involve several valuable aspects, and the following conditions are assumed to be essential for the accumulation of long oligonucleotides by both the RNA world hypothesis and hydrothermal origin of life hypothesis to be compatible. First, the oligonucleotide formation could have proceeded if it had started from higher oligonucleotides in the presence of Na^+-Mont. This assumption has been verified in a CL reaction at low temperatures, wherein activated nucleotide monomers could be continuously fed to the reaction mixture.[14,21,22] Thus, the possibility of the elongation of an oligonucleotide on a mineral surface should be experimentally verified at high temperatures as a future subject, although the mechanism and the length of the resulting oligonucleotides formed under hydrothermal conditions would still be unclear. Second, the oligonucleotides could not have survived in the absence of any additives if they were exposed to prebiotic hydrothermal vent conditions for a period longer than a few hours; further, it is known that some minerals inhibit the degradation of nucleotides.[53,54] Third, the formation of oligonucleotides could have proceeded if the 2-mer formation could be facilitated using additives that enhance the associate formation between two activated nucleotide monomers. These predictions would be useful for the future investigations of the oligonucleotide formation under hydrothermal conditions.[52,58,71]

Conclusions

The kinetics of the oligo(C) formation reaction from ImpC in the presence of Na^+-Mont was investigated at 0−100 °C. The rate constants for the formation of oligo(C) in the presence of Na^+-Mont increased in the order 2-mer ≪ 3-mer < 4-mer. It was deduced for the first time that the associate formation between an activated nucleotide monomer and an elongating oligonucleotide would be based on the interaction between the two reactants at the phosphoester and/or ribose moieties rather than at the nucleotide bases. The activation parameter analysis showed that the 2-mer formation is disadvantageous for $\Delta S^{\ddagger}$. The main reason for the decrease in the yield of oligo(C) with temperature is that the relative rate of the formation of the 2-mer decreases with temperature. It was confirmed that the effect of the degradation of oligo(C) on Na^+-Mont is relatively low. These analyses provided insights into the chemical evolution of RNA for the RNA world hypothesis and hydrothermal origin of life hypothesis to be compatible with each other.

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Supporting Information Available: Tables of reaction curves for the oligo(C) formation at 0−100 °C. This material is available free of charge via the Internet at http://pubs.acs.org

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