J. Mol. Evol. 14, 243–250 (1979)

Journal of Molecular Evolution
© by Springer-Verlag · 1979

Self-Condensation of Activated Dinucleotides on Polynucleotide Templates With Alternating Sequences

R. Lohrmann and L.E. Orgel

The Salk Institute for Biological Studies, San Diego, California 92112, USA

Summary. We have prepared substantial quantities of the alternating polymers poly(U-G) and poly(C-A) and have used them as templates for the self-condensation of ImpApC, ImpCpA, ImpGpU and ImpUpG. We find that the condensation of ImpGpU and ImpUpG on poly(C-A) is efficient, the condensation of ImpCpA on poly(U-G) is moderately efficient, while the condensation of ImpApC on poly(U-C) proceeds poorly. In many cases, the product is predominantly 3'-5'-linked.

These reactions demonstrate unequivocally, for the first time, that template-directed reactions occur in double-helical structures. Furthermore, they describe for the first time a pair of reactions in which each of two complementary polymers facilitates the synthesis of the other. The prebiotic significance of these findings is discussed.

Key words: Template-directed condensation – Phosphorimidazolides –Oligonucleotides with alternating sequence – Linkage isomerism – Prebiotic

Abbreviations: Im, imidazole; MeIm, 1-methylimidazole; EDTA, ethylenediamine tetraacetic acid; A, adenosine; C, cytidine; G, guanosine; U, uridine; T, thymidine; Xp (X = A, C, G or U), nucleoside 3'-phosphate; pX, nucleoside 5'-phosphate; XTP, nucleoside 5'-triphosphate; AppA, P_1,P_2-diadenosine 5'-diphosphate; XpY (X,Y = A, C, G, or U), dinucleoside phosphate e.g. ApC with X = A, Y = C, adenylyl - [3'-5']-cytidine; pXpY, dinuleotide with a 5' -phosphate terminal; $(pXpY)_n$ (n = 2,3...), oligomer of pXpY; (Ap)n (n = 2,3...), oligomer of Ap; ImpX, 5'-phosphorimidazolide of a nucleoside; ImpXpY, 5'-imidazolide of pXpY; poly (X-Y), polyribonucleotide with alternating sequence; poly d (X-Y), polydeoxyribonucleotide with alternating sequence; ODU, optical density unit measured at 260 nm; BAP, bacterial alkaline phosphatase E. coli. A star above the symbol for phosphate indicates the position of the radioactive label; P_i, inorganic phosphate.

0022–2844/79/0014/0243/$ 01.60

Introduction

In our early work (Orgel and Lohrmann, 1974) we showed that a poly(U) template catalyzes the synthesis of oligoadenylates from ImpA and that a poly(C) template catalyzes the corresponding reaction of ImpG (Lohrmann and Orgel, 1978). However, the converse reactions, the synthesis of oligo(U)'s from ImpU on a poly(A) template or of oligo(C)'s from ImpC on a poly(G) template, do not occur. Thus our early work failed to demonstrate the possibility of non-enzymatic nucleic-acid replication in two important respects. First, we did not demonstrate the incorporation of pyrimidine nucleotides into oligomers. Second, we worked only with three-strand helices, 2 poly(U)-ImpA and 2 poly(C)-ImpG and did not demonstrate reactions in a double-stranded helix.

In a recent paper we described a new approach to this problem. We showed that random copolymers containing purines and pyrimidines would facilitate the incorporation of pyrimidine-containing dinucleotides into oligomers, e.g. the incorporations of pUpG into GpGpUpG or pApApApApUpG (Ninio and Orgel, 1978). Here we study in greater detail the incorporations of ImpUpG, ImpGpU, ImpCpA and ImpApC into oligomers in the presence of strictly alternating poly(U-G) and poly(A-C) templates. In these cases there is no possibility of triple-helix formation, so our experiments demonstrate unambiguously the incorporation of pyrimidine nucleotides into oligomers in double-stranded helical structures.

Alternating RNA can be synthesized enzymatically only in a very small amount, so the synthesis of radioactive imidazolides and the template reaction had to be carried out on a very small scale. This necessitated some modification of our standard procedures (Lohrmann and Orgel, 1979).

Experimental

Materials

Reagent grade chemicals were used throughout. 1-Methylimidazole (Aldrich) was purified by redistillation. Nucleoside 5'-triphosphates and 3'-5'-linked dinucleoside phosphates GpU, UpG, CpA, and ApC were purchased from Sigma, alternating oligonucleotides $d(pCpA)_{6\text{-}9}$ and $d(pGpT)_{6\text{-}9}$ from Collaborative Research, deoxynucleoside 5'-triphosphates from P-L Biochemicals and [^{32}P]-labelled nucleoside triphosphates from Amersham. Polynucleotide kinase was obtained from Miles Laboratories, RNase T_1 from Calbiochem, pancreatic RNase from Boehringer, bacterial alkaline phosphatase (BAPF grade) from Worthington, and DNA-polymerase (M. Luteus) from P-L Biochemicals. E. coli K12 cells were purchased from Grain Processing Corporation. RNA-polymerase (220,000 units) was prepared according to Burgess and Jendrisak (1975).

Chromatography and Electrophoresis

Paper chromotography was carried out on Whatman 3MM paper by the descending technique. The following solvent systems were used:

n-propanol, concentrated ammonia and water (55:10:35), System I
n-propanol, triethylamine and water (55:10:35), System II

95% ethanol, and 1M ammonium acetate made up to 2 x 10^{-3}M in EDTA and brought to pH 5.0 with glacial acetic acid (7:3), System III

Isobutyric acid, concentrated ammonia and water (66:1:33), System IV

Paper electrophoresis was performed on Whatman 3MM paper at 3000 volts (55 volts/cm) using varsol as a coolant. The following buffer was used:

0.03M potassium phosphate, pH 7.1, System V.

The chromatographic and electrophoretic mobilities of various relevant compounds are listed in Table 1.

Quantitative estimates of product yields were obtained by running paper chromatograms or electropherograms through a Baird Atomic RSC 363 scanner with integrator. Sometimes results from several chromatographic and electrophoretic systems were collated to estimate the yields of individual compounds. Yields are expressed as the

Table 1. Chromatographic and electrophoretic mobilities

Compound	System I[a]	System II[a]	System III[a]	System IV[a]	System V[b]
A	1.55	1.43	1.43	1.38	0.00
pA	1.00	1.00	1.00	1.00	1.00
Ap	1.12		0.88		1.06
Cp	1.16		0.97		1.23
Gp	0.92		0.80		1.14
Up	1.11		1.27		1.22
pAp	0.70		0.40		1.63
pCp	0.77		0.43		1.61
pGp	0.54		0.31		1.54
pUp	0.64		0.57		1.65
ATP	0.88		0.25	0.52	
AppA	1.14		0.52		0.78
ApC	1.24		0.91	1.24	0.43
CpA	1.27		0.96	1.14	0.47
GpU	0.92		1.15	0.54	0.46
UpG	1.01		1.10	0.53	0.49
pApC	0.77	0.83	0.45	0.78	1.12
pCpA	0.79	0.87	0.45	0.82	1.13
pGpU	0.57	0.85	0.46	0.27	1.08
pUpG	0.60	0.72	0.47	0.30	1.12
ImpApC	1.36	1.27			
ImpCpA	1.39	1.32			
ImpGpU	0.84	1.09			
ImpUpG	0.84	1.03			
$(pApC)_2$	0.38				
$(pCpA)_2$	0.38				
$(pGpU)_2$	0.20				
$(pUpG)_2$	0.26				
$(Ap)_2$	0.85		0.31		1.05
$(Ap)_4$	0.44		0.03		

[a] R_f values are given relative to pA

[b] Electrophoretic mobilities are given taking $R_{adenosine} = 0.0$ and $R_{adenylic\ acid} = 1.0$

percentage of the total radioactivity on the paper, after allowing for the background. In some of our degradation studies the amount of radioactivity on a chromatogram was too low to permit us to make reliable estimates of the isomer ratios using the strip scanner. In these cases, we cut out the radioactive zones and counted their radioactivity in a Beckman liquid scintillation counter.

Preparations

Dinucleotides. pCpA, pApC, pGpU and pUpG were synthesized from the corresponding dinucleoside monophosphates and $POCl_3$ (unpublished). The corresponding [^{32}P]-labelled $\overset{*}{p}$XpY's were synthesized from XpY with polynucleotide kinase and γ-[^{32}P]-ATP (5 Ci/mmole) (Richardson, 1965). The reaction mixture was diluted with the corresponding nonradioactive pXpY before it was chromatographed on Whatman 3MM paper in System IV. The $\overset{*}{p}$XpY containing zones were cut out, washed with absolute ethanol and subsequently eluted with H_2O. The aqueous solutions were evaporated in vacuo in presence of triethylamine. The final residues were dried and kept in vacuo over P_2O_5 and solid NaOH.

Imidazolides. The imidazolides ImpXpY were prepared by a modification of a published procedure (Mukaiyama and Hashimoto, 1971; Lohrmann and Orgel, 1979) as described in the following for ImpUpG: An anhydrous solution containing the triethylammonium salt of pUpG (300 ODU_{260}), Im (18 mg), trioctylamine (30 μl), dimethylsulfoxide (200 μl), dimethylformamide (200 μl), triphenylphosphine (30 mg) and 2,2'-dipyridyl disulfide (25.2 mg) was kept at room temperature for 1 h. An aliquot chromatographed in System II on Polygram CEL 300 UV_{254} (Brinkmann) showed that the reaction was complete. The reaction mixture was added dropwise to a mixture containing acetone (10 ml), diethylether (5 ml), triethylamine (0.5 ml) and $NaClO_4$ (150 μmole) under vigorous stirring. The white precipitate of the sodium salt of ImpUpG was collected by centrifugation, washed with acetone and later with diethylether. The imidazolide was dried and stored over P_2O_5 and solid NaOH, in vacuo. The yield of isolated material was better than 90%. Chromatography of an aliquot in System II showed it to be better than 98% pure, a trace of pUpG being the only UV-absorbing contaminant. Other ImpXpY's were prepared using essentially the same procedure. When kept in dilute aqueous solution (pH $\leqslant$ 7) they hydrolyzed completely to the corresponding pXpY's. In previous studies we established that no linkage isomerization occurs during the preparation (Lohrmann and Orgel, 1979).

Radioactive $\overset{*}{Imp}$XpY's were prepared from the freshly prepared $\overset{*}{p}$XpY's as in the following example: An anhydrous mixture containing the triethylammonium salt of $\overset{*}{p}$UpG (20 ODU_{260} ca. 20 μCi), Im (1.4 mg), trioctylamine (10 μl), dimethylsulfoxide (50 μl), dimethylformamide (50 μl), triphenylphosphine (6 mg) and 2,2'-dipyridyldisulfide (5 mg) was kept at room temperature. It was crucial for the success of the reaction to exclude every trace of moisture. After 2 h an aliquot was cochromatographed with nonradioactive ImpUpG on Polygram CEL 300 (Brinkmann). The radioactive zones were cut out and counted in a Beckman scintillation counter. $\overset{*}{Imp}$UpG had formed in 90% yield. To purify the imidazolide from slower moving unreacted $\overset{*}{p}$UpG (6%) and a faster moving unknown product, the reaction mixture was chromatographed on 3MM paper in System II. The imidazolide-containing spot was cut out while the chromatogram still retained some triethylamine, and eluted with a methanol-triethylamine-

water mixture (4:1:5). The eluate was evaporated to dryness in vacuo (10^{-2} mm Hg) at $< 5^{o}$ (Evapomix). It is important that triethylamine is always present during the evaporation. The material was kept under vacuum over P_2O_5 and solid NaOH for ca. 2 h at room temperature before it was used.

Polynucleotides. Strictly alternating poly d(C-A) and poly d(G-T) were prepared using $d(pGpT)_{6-9} \cdot d(pCpA)_{6-9}$ primers with DNA polymerase (M. Luteus) and the 4 deoxynucleoside 5'-triphosphates as substrate (Wells et al., 1965). In preliminary experiments with α-[^{32}P]-labelled dCTP and α-[^{32}P]-dATP we established, by nearest neighbour analysis that the sequence of the poly d(C-A) strand was strictly alternating. We prepared ca. 250 ODU's of unlabelled poly d(A-C)·poly d(T-G) DNA in a scaled-up reaction.

Polynucleotides were prepared with RNA polymerase in presence of the alternating double-stranded DNA (Nishimura et al., 1965; Morgan, 1970). The poly d(G-T) strand was transcribed when CTP and ATP were the substrates, giving poly (C-A). Preliminary experiments using α-[^{32}P]-labelled ATP or α-[^{32}P]-CTP and carrying out neighbor analysis on the 2 radioactive polymeric products established that the poly (C–A) was strictly alternating, and hence that the poly d(G-T) strand must also have been strictly alternating. Poly(U-G) was formed under similar conditions, when GTP and UTP were the substrates. We prepared ca. 170 ODU's of poly (C-A) and ca. 60 units of poly(U-G). Since prolonged dialysis of these preparations did not remove traces of nucleoside triphosphates and citrate buffer, each polymer was further purified by gel filtration on a Sephadex G150 column.

Template-Directed Condensations: General Procedure

A solution (5 μl, pH 7.5) containing 0.15M $MgCl_2$, 0.60M NaCl, 0.2M MeIm-HCl buffer and 3 ODU of [^{32}P]-Imp*XpY (ca. 5 μCi) and a complementary polynucleotide (3 ODU) as template was kept at 0°C. Toluene (5λ) was added to keep the solutions sterile. In control experiments the polynucleotide was omitted. Aliquots were taken at different times, mixed with EDTA Na_4 and chromatographed in System I for ca. 40 h. For results see Table 2.

Analysis of Reaction Products

Isomer Ratios. The dimers (tetranucleotides) formed in our reactions were analyzed by enzymatic degradation and chromatographic study of their radioactive fragments (see Lohrmann and Orgel, 1979 for more details). In the case of $(^{*}pCpA)_2$ and $(^{*}pApC)_2$ we treated the dimers first with BAP and chromatographed the incubation mixture in System III. The only radioactive spots were P^{i} and the tetranucleotides CpA*pCpA and ApC*pApC, respectively. The cpm ratio of P^{i}:tetranucleotide was in both cases 1:1. Each tetranucleotide was then exposed to RNase T_2, followed by chromatography in System I. From the ratio of radioactive mononucleotide to dinucleotide we calculated that the dimer *pCpA*pCpA contained 16% of the natural 3'-isomer *pCpA^{3}*pCpA and 84% of the 2'-isomer. Treatment of the dimer fragment A^{2}*pCp with 1N KOH (48 h, room temperature) and chromatography in System III showed that only one radioactive product formed which cochromatographed with Ap. RNase T_2 treatment of ApC*pApC gave C*p and C*pAp in the ratio of 4:1, indicating that 80% of the dimer $(pApC)_2$ contained the natural (3'-5')-linkage.

Table 2. Percentage yields and isomer distribution from condensation involving ImpXpY's in presence or absence of a polynucleotide template with alternating complementary base sequence

Template (reaction time)	Designation	ImpXpY	pXpY	Pyrophosphate	$(pXpY)_2$[a]	$(pXpY)_{3+}$[b]
+ poly (C-A) (8d)	X=G, Y=U	18.6	32.6	4.9	18.9(96)	25.0 (n=9.2)
- "		42.3	52.9	2.4	1.8	0.6
+ "	X=U, Y=G	19.5	32.6	4.0	24.2(65)	19.8 (n ⩾ 5.1)
- "		43.7	46.1	8.7		1.6
+ poly(G-U) (10d)	X=A, Y=C	15.4	77.8	1.0	5.4(80)	0.6
- "		32.6	65.2	1.9	0.3	
+ "	X=C, Y=A	5.4	64.6	2.0	23.2(16)	4.8 (n ⩾ 5.5)
- "		8.7	88.9	0.6	1.1	0.8

[a] The numbers given in parentheses after the yields give the percentage of the material that is [3'-5']-linked

[b] The number n, given in parentheses under the yields, gives the average chain length

In the case of $(\overset{*}{p}GpU)_2$ and $(\overset{*}{p}UpG)_2$ the dimers were directly treated with RNase T_1 and pancreatic RNase, respectively. The incubation mixtures were analyzed in System III. $(\overset{*}{p}GpU)_2$ was degraded to $\overset{*}{p}GpU\overset{*}{p}$ in 96% yield. The latter fragment was degradable with KOH to $\overset{*}{p}Gp$ and $U\overset{*}{p}$, in the ratio of 1:1. Similarly, $(\overset{*}{p}UpG)_2$ was degraded to $\overset{*}{p}UpG\overset{*}{p}$ in about 65% yield.

The pyrophosphates which chromatograph in System I between monomer and dimer remained unchanged when directly exposed to BAP. However, when incubated with RNase T_2 and then with BAP the radioactive end products were the expected P_1P_2-dinucleoside pyrophosphates, for instance, in the case of $C^5pA^5\overset{**}{p}p^5A^3pC$ all the radioactivity cochromatographed with AppA.

Estimate of Chain Length. The trimer and higher oligomers chromatographing near the origin were degraded by alkaline hydrolysis in 1N KOH (50 h, room temperature) and then analyzed in System III or V. The average chain length (Table 2) was estimated from the ratio of 5'-phosphonucleoside 2'(3')-phosphate to nucleoside 2'(3')-phosphate, taking into account that every second monophosphate was radioactively labelled.

Discussion

The data in Table 2 establish that poly(C-A) acts as a very efficient template for the self-condensation of ImpGpU and as a moderately efficient template for the self-condensation of ImpUpG. The efficiencies of these reactions are comparable with those obtained in ImpA self-condensations on poly(U) (Orgel and Lohrmann, 1974). The yields are smaller in the reciprocal condensations. ImpApC gives relatively small amounts of condensation product on poly(U-G), while ImpCpA gives a good yield of $(pCpA)_2$, but only 4.8% of $(pCpA)_3$ and higher oligomers. In preliminary experiments

performed under very similar conditions poly(U) and poly(C) had no effect on the self-condensation of ImpApC or ImpCpA.

The isomeric character of the newly synthesized nucleotide bonds is particularly interesting. The linkage in pGpUpGpU was almost entirely (96%) the natural 3'-5'-linkage. The 3'-5'-isomer of pUpGpUpG was also obtained as the major product (65%). On the other hand, while pApCpApC was 80% 3'-5'-linked, pCpApCpA was 82% 2'-5'-linked. We have no explanation of these isomer ratios.

The results described in this paper allow us to draw two important, new conclusions. First, efficient template-directed synthesis can occur in double helices. No triple helices are possible in the present system, since it is not possible to pair two purines with one pyrimidine in a manner that is isostructural with the 2U-A and 2C-G base triplets. This conclusion was strongly suggested by our results using random copolymers (Ninio and Orgel, 1978), but we could not previously exclude the possibility that limited lengths of triple helix were formed. Second, we have for the first time shown that each of a pair of complementary polymers can direct the synthesis of the other – this is not possible with monomers and homopolymers, since polypurines do not form organized helices with pyrimidine monomers.

Prebiotic Significance

The new conclusions that we draw from our data are of considerable importance in the context of prebiotic chemistry. Previously, the majority of our experiments have utilized the homopolymers poly(U) or poly(C) as template and derivatives of A or G as substrates (Orgel and Lohrmann, 1974). Under these special circumstances triple helices rather than double helices are formed. One could, therefore, still have questioned whether *base-pairing* was sufficient to permit non-enzymatic template directed synthesis. The present systems, because they cannot form triple-strand helices, show unambiguously that condensation does occur in double-stranded structures.

Our results also emphasize the importance of short oligomers for the origin of template-directed reactions (Ninio and Orgel, 1978). While template-directed reactions are possible with monomers, exponential increase in the amount of condensed material is not. Such an increase is possible only if each of a pair of complementary oligonucleotides can act as templates for the synthesis of the other. This does not occur in homopolymer systems, since poly(A) does not act as a template for oligo(U) synthesis, etc.

Finally, the isomer ratio that we obtain shows that certain combinations of donor and acceptor give very high ratios of [3'-5']- to [2'-5']-linked product. If Usher's conclusion with regard to the hydrolytic stability of 2'-5'- and 3'-5'- linked products holds generally (Usher and McHale, 1976), a subtle interplay between rate of formation and rate of hydrolysis must determine which base sequences and which linkages within such sequences would be favored by natural selection.

Acknowledgements. This work was supported by NASA grant No. NGR 05-067 and NIH grant No. GM 13435. We would like to thank R.D. Wells for valuable advice on the preparation of polynucleotides with alternating sequence. We thank A.R. Hill and J.E. Tomaschke for their technical assistance, and Ms. R. Brown for manuscript preparation.

References

Burgess, R.R., Jendrisak, J.J. (1975). Biochemistry **14**, 4634–4638
Lohrmann, R., Orgel, L.E. (1978). In: Origin of life. Proceedings of the Second ISSOL Meeting–Fifth ICOL Meeting (H. Noda, ed.), Center for Academic Publications Japan/Japan Scientific Societies Press 235–244
Lohrmann, R., Orgel, L.E. (1979). J. Mol. Evol. **12**, 237–257
Morgan, A.R. (1970). J. Mol. Biol. **52**, 441–446
Mukaiyama, T., Hashimoto, M. (1971). Bull. Chem. Soc. Japan **44**, 2284
Ninio, J., Orgel, L.E. (1978). J. Mol. Evol. **12**, 91–99
Nishimura, S., Jones, D.S., Khorana, H.G. (1965). J. Mol. Biol. **13**, 302–324
Orgel, L.E., Lohrmann, R. (1974). Accounts of Chem. Res. **7**, 368–377
Richardson, C.C. (1965). Proc. Nat. Acad. Sci. USA **54**, 158–165
Usher, D.A., McHale, A.H. (1976). Proc. Nat. Acad. Sci. USA **73**, 1149–1153
Wells, R.D., Ohtsuka, E., Khorana, H.G. (1965). J. Mol. Biol. **14**, 221–240

Received June 12, 1979