COMPLEMENTARILY ADDRESSED MODIFICATION OF rRNA WITH *p*-(CHLOROETHYLMETHYLAMINO)BENZYLIDENE HEXANUCLEOTIDES

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1. Introduction

Methods for highly specific chemical modification of nucleic acids are of interest with respect to the elucidation of nucleic acid structure and functioning. The present paper deals with a new approach to specific modification of nucleic acids at definite bases in the desired region of the polynucleotide chain ("complementarily addressed modification"). This is a chemical modification within a complementary complex of a nucleic acid with a special addressing reagent containing both some modifying group and an oligonucleotide part complementary to the desired region of the nucleic acid molecule (fig. 1). This concept has been discussed earlier by Grineva and Knorre [1, 2]. Some alkylating derivatives of oligonucleotides and of tRNA have also been described [3–5].

We have found that alkylating derivatives of hexaadenylate and hexanucleotides from tRNA digests – *p*-(chloroethylmethylamino)benzylidene hexanucleotides (fig. 1) – are complementarily bound to rRNA and that they do alkylate rRNA quantitatively, within the complex, whereas without binding alkylation proceeds to an extremely small extent. The specificity of such a modification obviously depends on the complementary properties of oligonucleotides.

The application of complementarily addressed alkylation to achieve highly specific chain scission of both DNA and RNA at alkylated guanine residues [6, 7] seems to us to give a new general approach to the study of nucleic acid structure.

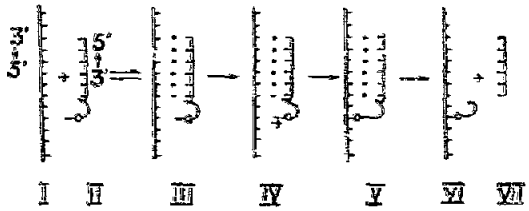
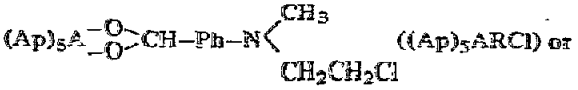
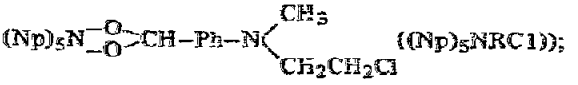


Fig. 1. Scheme of complementarily addressed alkylation.
I – Fragment of nucleic acid chain, here rRNA; II – addressing reagent:

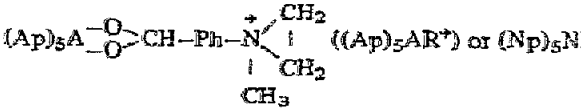


((Ap)$_5$ARCl) or



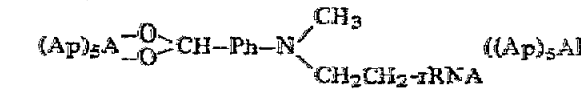
((Np)$_5$NRC1));

III – complementary complex of nucleic acid-reagent;
IV – complex with active reagent intermediate:



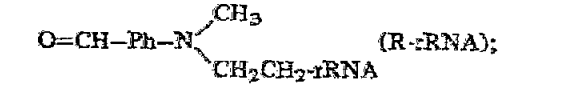
((Ap)$_5$AR$^+$) or (Np)$_5$NR$^+$;

V – alkylated rRNA:



((Ap)$_5$AR-rRNA)

VI – alkylated rRNA treated at pH 4:



(R-rRNA);

VII – addressing oligonucleotide.

2. Materials and methods

Hexaadenylate obtained from poly A [3] was kindly supplied by Dr. Shamovsky (of this institute); $(Np)_5N$ is a mixture of hexanucleotides that was prepared by alkaline phosphatase dephosphorylation of $pN(pN)_5$. The latter was isolated by Dr. Kozorovitzy (of this institute) from tRNA (yeast) digested with non-specific 5'-endonuclease from cobra venom [8]. rRNA was isolated from *E. coli* MRE 600 by phenol extraction and ethanol precipitation. From polyacrylamide gel electrophoresis data the sample mainly contained 16 S and 23 S RNA. [^{14}C]*p*-(Chloroethylmethylamino)benzylidene hexanucleotides were obtained by a slight modification of the general procedure of Zarytova et al. [3]. The samples contained 80–95% $(Ap)_5ARCl$ or $(Np)_5NRCl$. $(Ap)_5ARCl$: λ_{max}^{pH7} 257 nm, λ_{max}^{pH10} 259 nm, ϵ_{260}^{pH7} 110×10^3, calculated ϵ_{260}^{pH7} 89.7×10^3. R_f relative to pA (*n*-propanol–NH_3–water, 50:10:35) is 1.18; $(Np)_5NRCl$: λ_{max}^{pH7} 256, λ_{max}^{pH13} 260 nm, ϵ_{260}^{pH7} 84.5×10^3.

The conditions of reagent and oligonucleotide binding with rRNA were 0.2 M NaCl, 0.01 M $MgSO_4$, 0.01 M Tris-HCl, pH 7.3 (buffer 1) at 0°. The complexes were isolated by gel filtration on Sephadex G-75 (46 × 0.8 cm) in the same buffer at 0° according to [8]. The first peak contained the complexes, the second one the excess reagents. The complex is disrupted by heating at 40° (pH 4) and further gel filtration at 45° in 0.02 M Tris-HCl pH 7.2, (buffer 2).

rRNA was alkylated with reagent without binding by incubation at 40° in buffer 2. For alkylation within the complex incubation was carried out at 5, 10 or 20° in buffer 1 after complex isolation at 0° or after constituents were mixed prior to incubation. Alkylation was stopped by reagent hydrolysis to *p*-(chloroethylmethylamino)benzaldehyde (RCl) and oligonucleotides at pH 4, 40°, 30 min according to [9]. Alkylated rRNA (R-rRNA, fig. 1) was isolated by gel chromatography on Sephadex G-75 (46 × 0.8 cm) at 4–5° in buffer 2.

The extent of rRNA alkylation was estimated from the radioactivity of the polymer fraction after gel filtration at 45° (using a Nuclear Chicago Mark I scintillation counter). The extent of non-covalent binding was measured by the radioactivity of the polymer fraction after gel-filtration at 0°. The results were expressed in moles of reagents bound to the sum of

Table 1
Hexanucleotide and *p*-chloroethylmethylaminobenzylidene hexanucleotide binding with rRNA at 0°.

Reagent	Initial solution		Complex
	$[rRNA]_0$ μM	$\frac{[reagent]_0}{[rRNA]_0}$	$\frac{[reagent] \text{ in complex}}{[rRNA]}$
[^{14}C](Ap)$_5$ARCl	0.60	9	8
	0.60	19	16
	0.43	40	32
	0.11	55	42
	0.78	276	46
$(Ap)_5A$	1.62	18	17
	1.61	48	42
	1.58	156	50
	1.51	465	52
[^{14}C](Np)$_5$NRCl	1.48	320	40
	1.24	450	58
$(Np)_5N$	0.64	600	80

0.2 M NaCl, 0.02 M $MgSO_4$, 0.01 M Tris-HCl, pH 7.3.

16 S and 23 S RNA (ϵ_{260}^{pH7} 33×10^6). The extent of reagent ionized in the course of alkylation (moles of the active alkylating intermediate formed in the rate determining step of the reaction) was calculated from equation [9, 10] $[(Ap)_5AR^+] = [(Ap)_5ARCl]_0 (1-e^{-k_0 t})$, where $k_0^5 = 0.46 \times 10^{-6}$, $k_0^{10^\circ} = 1.41 \times 10^{-6}$, $k_0^{20^\circ} = 6.3 \times 10^{-6}$ and $k_0^{40^\circ} = 1.14 \times 10^{-4}$ sec^{-1}. The values of k_0 were shown to be practically indepenuent of the structure of the oligonucleotide part of the reagent [9]. The extent of hexanucleotide binding with rRNA was measured by absorbance at 260 nm of the first peak after complex dissociation brought about by gel filtration at 45° according to [8]. Acid hydrolysis of $(Ap)_5AR$-rRNA in 0.5 N HCl at 100° and identification of alkylation products were carried out according to [11, 12].

3. Results

Alkylating derivatives of hexaadenylate, $(Ap)_5ARCl$, can be bound to rRNA as can $(Ap)_5A$ itself. Table 1 shows the extent of binding $(Ap)_5ARCl$ and $(Ap)_5A$ to rRNA in the presence of Mg^{2+} with various excess amounts of the reagent. Saturation is nearly achieved

Table 2
rRNA alkylation with *p*-chloroethylmethylaminobenzylidene hexanucleotides; rRNA concentration 0.04–0.2 μM.

Reagent	$\frac{[\text{Reagent}]_0}{[\text{rRNA}]_0}$	t°	(hr)	Calculated extent of reagent ionization in the course of incubation: $(Ap)_5AR^+$/rRNA	Moles of R in R-rRNA	Proportion of $(Ap)_5AR^+$ alkylating rRNA (%)
1	2	3	4	5	6	7
1. Alkylation within the complex after complex isolation						
$(Ap)_5ARCl$	37	10	307	29.4	25	86
	52	10	216	35.0	29	84
	45	20	30	23.8	22	92
$(Np)_5NRCl$	40	10	240	28.0	28	100
	58	10	168	33.5	30	90
2. Alkylation in a mixture of constituents without previous complex isolation						
$(Ap)_5ARCl$	14	5	768	10	9.8	97
	28	5	792	20.5	20.0	98
	35			25.5	24.7	97
	56			41	32.3	79
	17	20	168	17	15	89
	28.5			28.5	26	92
	107			107	31	30
	214			214	32	15
	29.5		61	22	20	91
3. Alkylation without complex formation						
$(Ap)_5ARCl$	46	40	12	46	2	4

with a two-fold excess of $(Ap)_5A$ or $(Ap)_5ARCl$. But a ten-fold excess of $(Np)_5NRCl$ does not cause saturation. According to Dr. S.K. Vasilenko's data (this laboratory) a 20-fold excess of $pN(pN)_5$ concentration is necessary to saturate rRNA. Heating at 40–45° disrupts the complexes to their constituents, which can be separated by gel filtration at 45°.

Apparent association constants, K_{ap}, can be estimated on the assumption that reagent binding with different regions of rRNA proceeds independently and with approximately the same K_{ap}. In the case of binding at 0° the number of binding sites is about 50; hence $[\text{binding sites}] = 50[\text{rRNA}]_0$ and

$$K_{ap} = \frac{[\text{reagent}]_{bound}}{(50[\text{rRNA}]_0 - [\text{reagent}]_{bound})([\text{reagent}]_0 - [\text{reagent}]_{bound})}\ M^{-1}$$

K_{ap} of $(Ap)_5ARCl$ and $(Ap)_5A$ are nearly the same order of magnitude ($10^5\ M^{-1}$). The similar binding abilities of $(Ap)_5A$ and $(Ap)ARCl$ accord closely with the stability of three stranded complexes of poly U with both oligoadenylates and their alkylating derivatives [13].

Incubation of $(Ap)_5ARCl$ with rRNA results in covalent binding of the reagent to rRNA; the reagent remains bound after gel filtration at 45°. Alkylation proceeds to the formation of alkylated rRNA, $(Ap)_5AR$-rRNA (fig. 1), which transforms to R-rRNA at pH 4 and 40° due to acid hydrolysis of the benzylidene linkage in the reagent residue.

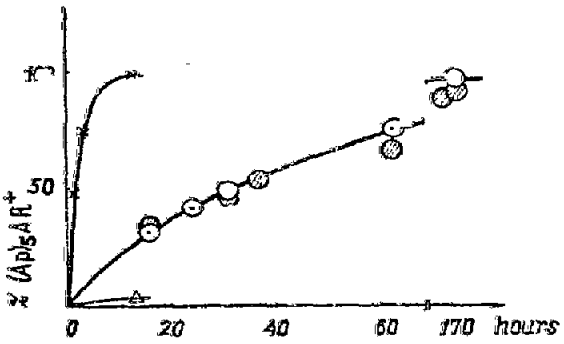
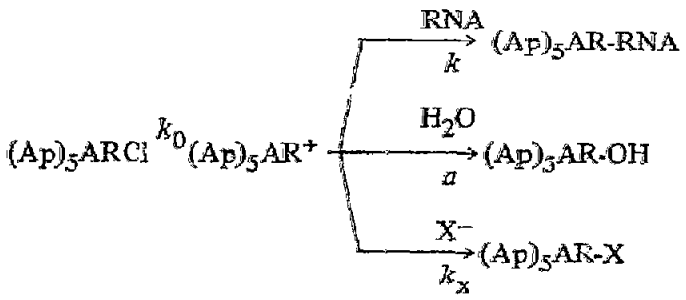


Fig. 2. Plots of $(Ap)_5AR^+$ formation and of rRNA alkylation with $(Ap)_5AR^+$. △ – Alkylation at 40° without complex formation; ● – alkylation at 20° within the complex; ○,× – $(Ap)_5AR^+$ formation at 20° and 40°, respectively, according to [10, 11].

According to [10, 14] the mechanism of rRNA alkylation with $(Ap)_5ARCl$ and $(Np)_5NRCl$ as aromatic chloroethylamines may be represented as follows.



(where X^- represents buffer nucleophiles and k_0, k, a, k_x the reaction rate constants.) The rate determining ionization of the C–Cl bond of the reagents results in an active ethylene immonium cation (compare [15]); in our case it is $(Ap)_5AR^+$ or $(Np)_5NR^+$ (fig. 1). Therefore the reaction of the reagent with RNA depends strongly on the ratio $k/(a+k_x)$ (reagent efficiency [9, 16].

Without complex formation the reactivities of the reagents with tRNA are known to be very poor, less than 1% at the usual (0.3–5 mM) tRNA concentration [9, 16]. rRNA (0.08 μM) takes up 4% $(Ap)_5AR^+$ at 40° in the absence of Mg^{2+} (table 2); reagent efficiency is 256 M^{-1}.

Alkylation within the complex results in nearly quantitative covalent binding of $(Ap)_5AR^+$ and $(Np)_5NR^+$ to rRNA. This may be seen from the proximity of the calculated values of the extent of

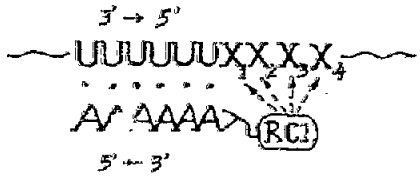


Fig. 3.

ionization of the reagents during incubation and the number of moles of reagents covalently bound to rRNA (table 2, columns 5–7). The rate of rRNA alkylation with $(Ap)_5ARCl$ coincides with the ionization rate of $(Ap)_5ARCl$ (fig. 2). These results mean that rates of by-reaction are immeasurably low in this case and reagent efficiency for alkylation within the complex is several orders of magnitude greater than that for alkylation without binding.

Table 2 shows that almost quantitative alkylation also occurs in the mixture of rRNA and reagent at 5° and 20° if the complex is not previously isolated. The reaction proceeds quantitatively during incubation up to the maximum extent of $(Ap)_5ARCl$ ionization to $(Ap)_5AR^+$. Small extents of reaction are only obtained with some excess of reagent over the number of binding sites.

Therefore it may be concluded that a large extent of reaction indicates that alkylation occurred within the complex. Alkylation within the complex proceeds even at 20°; complex concentration is rather high (about 90%) and K_{ap} is 10^5 M^{-1}; thus there is complementary binding in the complex (compare with [17, 13]).

Nearly quantitative alkylation within the complex suggests that almost every region of rRNA with binding site can be alkylated. Acid hydrolysis data show that main alkylated bases in R-rRNA modified at 5° within the complex: 16 S + 23 S RNA + 29 moles of $(Ap)_5ARCl$ are 7-alkylguanine (53%) and 3-alkylcytosine (21%).

4. Discussion

Quantitative alkylation of rRNA within the complex means that the alkylating group of the reagent attacks several bases that are adjacent to the 5'-terminus of binding site, one of which must be guanine or cytosine (fig 3). Accessibility of three or even two adjacent bases to the alkylating group of the reagent is suffi-

cient to allow more than 90% alkylation within the complex because the probability of guanine and cytosine occurrence in any position of rRNA is 0.33 + 0.21, according to the nucleotide composition of rRNA [19] and because the probability of the occurrence of nearest uridines is rather low.

Studies of Stuart–Brigleb models showed that even in the perfect double helix conformation of RNA–$(Ap)_5$ARCl complex the alkylating group could react with three of the nearest bases, namely, X_2, X_3 and X_4, but not X_1 [20]. The relationship between the bases alkylated and model studies allows one to assume that X_2 and X_3 bases are predominantly alkylated within the complex. Guanine seems to react quantitatively if X_2 and X_3 are guanine and cytosine or cytosine and guanine, respectively, because guanine reactivity is 7–10 times as great as that of cytosine [11, 12].

Binding of 50 moles of $(Ap)_5$A or $(Ap)_5$ARCl with rRNA at 0° does not mean that there are the same number of hexa U sequences in the rRNA. Complementarily binding can be true pairing, pairing with bulges and three stranded complex formation [21, 22]. Besides the decrease of RNA saturation with A_6RCl at 20° to 30 moles can be taken to indicate that $(Ap)_5$ARCl at 0° interacts with oligo U sequences from U_3 to U_6. Some decrease of saturation with increasing temperature indicates disruption first of the weakest binding. Thus, there is a possibility of increasing alkylation specificity to somewhat more than 0.5–1% modification at certain definite points of the chain. Some of the heterogeneity of alkylation specificity is connected with the nature of complementary binding.

Acknowledgements

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