AN UNSPECIFIC RNA (ADENINE-1-)METHYLASE FROM THE DINOFLAGELLATE *CRYPTHECODINIUM COHNII*

Edgar WERNER, Peter KAHLE, Ursula LANGE and Hans KRÖGER
Robert Koch-Institut, 1 Berlin 65, Nordufer 20, West Germany

Received 24 May 1975

1. Introduction

In eucaryotic organisms methylated nucleotides have been found in tRNA [1], rRNA [2], LnRNA [3], and mRNA [4]. Judging from the sequenced parts of tRNA [5] and rRNA [6,7], the attachment of methyl groups to RNA is a very specific process tRNA methylases retain their specificity when tested in vitro: no RNA except for heterologous tRNA is accepted and methylation takes place at positions where tRNA precursors are methylated in vivo. rRNA methylases, less well studied, are reported to require heterologous RNA in vitro, too [10,11].

The RNA methylase presented here does not fulfill these criteria. It is widely unspecific in vitro and so we do not know its function in vivo.

The source of the enzyme is the dinoflagellate *Crypthecodinium cohnii*. Dinoflagellates are reported to contain the most primitive nucleus observed in eucaryotes, particularly as to the chromosomal structure, which resembles that of bacteria [12].

2. Materials and methods

Enzyme preparation: *Crypthecodinium cohnii* (strain GC) was cultivated in the AXM medium of Provasoli and Gold [13]. Cells of the early stationary phase were harvested by centrifuging, washed in 12% (w/v) sorbitol, 0.004 M EDTA pH 7.0, and then suspended in 4 vols 0.06 M Tris maleate, pH 6.1, 0.005 M $MgCl_2$, 0.001 M dithioerithreitol (DTE) mixed with 5% (w/v) Dextran T 40 (Pharmacia, Frankfurt/M), 20% (v/v) glycerol, 1 M sorbitol (H-medium). The cells were homogenized similar to the method of Bhargava and Halvorson [14]: 2 × 40 ml were frozen in a French cell by placing it in an ethanol/dry-ice bath for about 8 min and then pressed at 20 000 psi. The procedure was repeated once. After thawing, the homogenate was diluted by half with H-medium, and centrifuged through a sucrose cushion of 44% (w/w) sucrose in 0.04 M Tris–HCl pH 7.6, 0.005 M $MgCl_2$, 0.001 M DTE (buffer A) at 23 000 *g* for 45 min. The sediment was washed with 32 ml 30% (w/w) sucrose in buffer A, resuspended in 16 ml 10% (w/w) sucrose in buffer A containing 0.6 M KCl, and then centrifuged at 145 000 *g* for 60 min. The supernatant was dialyzed against 0.01 M Tris–HCl, pH 7.6, 0.001 M EDTA, 0.005 M mercaptoethanol, 0.2% Triton X-100 (buffer B). The precipitate formed was spinned down at 44 000 *g* for 10 min. The enzyme solution thus obtained (about 23 ml) was adsorbed on to a TEAE-cellulose column (1.6 × 2 cm), equilibrated with buffer B, and washed with 15 ml 0.1 M KCl in buffer B. The enzyme was eluted with 8 ml 0.33 M KCl in buffer B. The enzyme fraction was dialyzed against 0.1 M Tris–HCl, pH 7.6, 0.001 M EDTA, 0.005 M mercaptoethanol, 0.2% Triton X-100 (buffer C), then adsorbed on to a phosphocellulose column (0.9 × 5 cm) equilibrated with buffer C. After washing with 15 ml buffer C elution was performed with 22 ml of a linear gradient from 0–0.6 M KCl in buffer C. The active fractions (about 7 ml) were pooled, concentrated by dialyzing against dry polyethyleneglycol to about one tenth volume, and finally dialyzed against buffer B without Triton X-100.

The standard assay mixture contained in a final volume of 0.15 ml: 17 μM S-adenosyl Me-[^{3}H] methionine (52 mCi/mmol), 110 mM Tris–HCl, pH 7.8, 0.5 mM dithioerythritol (DTE), 0.6 A_{260}

polynucleotide (0.1–0.2 A_{260} in the case of viral RNA), and 8 μg enzyme protein. The reaction mixture was incubated for 30 min at 30°C. RNA was precipitated by cetyltrimethylammonium bromide and prepared for scintillation counting as described previously [15].

Ribosome preparation from *Sacch. cerev.* and *Crypthec. cohnii:* The cells were homogenized in 50 mM Tris–HCl pH 7.5, 25 mM KCl, 5 mM $MgCl_2$ (TKM). The mitochondria-free supernatant of the homogenate was centrifuged 90 min at 100 000 *g*. The resulting pellet was washed in 0.5% X-100 in TKM. After sedimentation through a 0.5 M sucrose cushion the pellet was suspended in 50 mM Tris–HCl pH 7.6, 1 mM $MgCl_2$.

rRNA, where indicated, was prepared after Kumar [16], tRNA from *Crypthec. cohnii* was prepared from the 100 000 *g* supernatant by phenolization and subsequent passage through Sephadex G-100.

3. Results and discussion

The enzyme activity is extracted from a crude nuclear fraction by 0.6 M KCl or 0.5% Triton X-100. In both cases the sole reaction product is 1-methyladenine as demonstrated by base analysis [15]. Of the radioactivity recovered from the TLC plates (1000 cpm typically) 95% or more moved with 1-methyladenine, 2–4% with 6-methyladenine. The spots of 2-methyladenine, 6-dimethyladenine, 3-methylcytosine, 5-methylcytosine, 1-methylguanine, 2-methylguanine, 2-dimethylguanine, 7-methylguanine, 5-methyluracil together contained 1% or less. Radioactivity coincident with 1-methyladenine moved with 6-methyladenine after heating in 25% ammonia. The 2–4% 6-methyladenine supposedly have been formed from 1-methyladenine during incubation and/or recovery of RNA in media of pH 8–9.5% [17]. No methanol was liberated from RNA on hydrolysis in $HClO_4$, ruling out ribose methylation [18]. This methylation pattern holds true for all the polyribonucleotides tested (table 1). A series of rRNAs is accepted as substrate. Even into the homologous rRNA of *Crypthec. cohnii* methyl-groups are incorporated at a similar rate. 5S RNA, which has never been found to be methylated in vivo, is a substrate as well as viral RNAs and poly A. The tRNA values are lower for reasons unknown. DNA, both single stranded or double stranded, is not methylated.

The unspecificity indicated by these data, especially the methylation of poly A, suggested that

Table 1
Activity against various polynucleotides

Nucleic acid added	pmoles CH_3 incorporated
None	0.6
rRNAS	
E. coli[b]	14.6
P. aeruginosa[b]	18.8
B. stearotherm.[b]	14.3
Crypthec. cohnii[a]	13.6
16s	11.4
25s	13.4
Sacch. ceriv.[a]	19.1
rat liver[a]	10.1
5S	
E. coli[b]	12.2
B. stearoth.[b]	5.3
Sacch. ceriv.[b]	10.4
tRNA	
E. coli[c]	2.5
rat liver[a]	4.0
viral RNA	
AMV[d]	15.5
Qß[e]	7.6
MS2[e]	8.2
DNA, calf thymus[f]	
native	0.6
denatured	0.8
Poly A[g]	9.5
Ribosomes	
E. coli[b]	33.0
Crypthec. cohnii[h]	17.5
Sacch. ceriv.[h]	13.2

The pmoles CH_3 incorporated represent reaction rate measured. The amount of polynucleotides per assay was 0.6 A_{260} except for the viral RNAs, where it was 0.1–0.2 A_{260}. In the assays with ribosomes $MgCl_2$ was added to 0.16 mM. Sources of the polynucleotides: [a] prepared according Kumar [16]; [b] Dr V. Erdmann, Berlin; [c] Boehringer, Mannheim; [d] Dr K. Mölling, Berlin; [e] Miles Lab., Lausanne; [f] Dr U. Hagen, Karlsruhe; [g] Calbiochem, Luzern; [h] see Materials and methods.

Table 2
Extent of methylation with the RNAs from ribosomes of *Sacch. ceriv.*

Type of RNA	Molecules CH_3-/molecule RNA	Adenine residues present in the RNA
5S	2.9	30
18S	96	452
26S	122	871

0.4 μg of each RNA were incubated with increasing amounts of protein (up to 30 μg) as described in Materials and methods. Calculations of methylation extent are based on the plateau values of methyl incorporation and on the estimated chain length and base composition of the respective RNA [7,19,20].

besides an adenosine little is required for a methylation reaction to proceed on the polynucleotide. This is supported by the high extent of methylation measured with the RNAs from yeast ribosomes (table 2). The maximal density is reached in the 18S molecule by a ratio of 1 methylgroup to five adenine residues. Such a high degree of methylation has never been attained by the methylases reported on before. Nor has it been detected in any RNA.

Two attempts were made to narrow down the specificity of the enzyme. First we tested if strengthening the secondary structure of the RNA could suppress homologous methylation (fig.1). Increasing concentrations of K^+, Mg^{2+}, and spermidine, ions known to stabilize the conformation of RNA, were added and the resulting activities towards heterologous and homologous rRNA and tRNA compared. However, with both substrates the activities declined the same way. Second, ribosomes were added instead of rRNA to see if masking by proteins prevented unspecific methylation. This was found not to be the case. Ribosomes were a very active substrate (table 1).

It seems very impropable that the enzyme acts in vivo as it does in vitro. There should be means by which unspecific methylation is prevented. These may be a specific environment of soluble substances not yet imitated in the reaction tube, cofactors, attachment of the enzyme to some structure.

Considering the unspecificity, one cannot tell the physiological substrate. The enzyme may be a methylase of rRNA or of any other methylated RNA. However, it does not appear to be a tRNA methylase, because its unspecificity in vitro is in contrast to all known tRNA methylases. To exclude other RNAs as physiological substrate, one way will be the analysis of the RNAs of *Crypthec. cohnii* for 1-methyladenine.

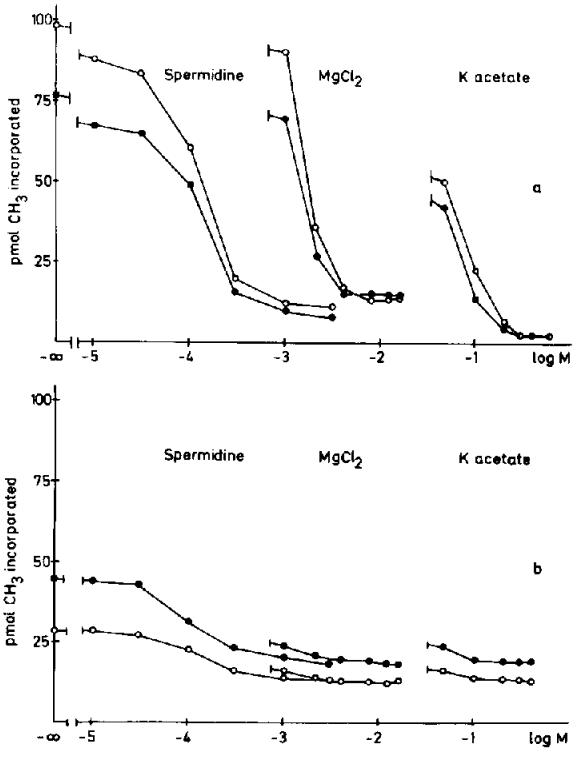

Fig.1. Influence of K^+, Mg^{2+} and spermidine on the reaction rate with (a) rRNA and (b) tRNA from *E. coli* (○——○) and *Crypthec. cohnii* (●——●) using 0.6 A_{260} RNA per assay. Sources of rRNA see legend to table 1, *E. coli* tRNA from Boehringer, Mannheim; *Crypthec. cohnii* tRNA see Materials and methods

Regardless of its physiological role, the enzyme is interesting for two reasons. Firstly, one may use it as a tool in the study of RNA functions by modifying these RNAs under much milder conditions than chemical methods allow. Secondly, the study of this enzyme may elucidate a methylation mechanism quite different from the known tRNA methylation, since tRNA methylases are by themselves specific wherease this enzyme is not.

Acknowledgements

We are indebted to Drs K. Mölling, V. Erdmann, and U. Hagen for giving us nucleic acid samples. This work was supported in part by the Deutsche Forschungsgemeinschaft and the Wissenschaftsministerium. The paper is dedicated to Professor H. J. Staudinger (Univeristy Giessen) on occasion of his 60th birthday.

References

[1] Iwanami, Y. and Brown, G. M. (1968) Arch. Biochem. Biophys. 124, 471–482.
[2] Iwanami, Y. and Brown, G. M. (1968) Arch. Biochem. Biophys. 126, 8–15.
[3] Reddy, R., Ro-Choi, T. S., Henning, D., Shibata, H., Choi, Y. C. and Busch, H. (1972) J. Biol. Chem. 247, 7245–7250.
[4] Perry, R. P. and Kelly, D. E. (1974) Cell 1, 37–42.
[5] Staehelin, M. (1971) Experientia 27, 1–11.
[6] Fellner, P., Ehresmann, C. and Ebel, J. P. (1972) Biochimie 54, 853–900.
[7] Klootwijk, J. and Planta, R. J. (1973) Eur. J. Biochem. 39, 325–333.
[8] Kuchino, Y. and Nishimura, S. (1970) Biochem. Biophys Res. Commun. 40, 306–313.
[9] Baguley, B. C. and Staehelin, M. (1968) Eur. J. Biochem. 6, 1–7.
[10] Sipe, J. E., Anderson, W. M., Remy, C. N. and Love, S. H. (1972) J. Bacteriol. 110, 81–91.
[11] Culp, L. A. and Brown, G. M. (1970) Arch. Biochem. Biophys. 137, 222–230.
[12] Giesbrecht, P. (1962) Zentralbl. Bakt. I. Abt. Orig. 187, 452–497.
[13] Provasoli, L. and Gold, K. (1962) Arch. Mikrobiol. 42, 196–203.
[14] Bhargava, M. M. and Halvorson, H. H. (1971) J. Cell. Biol. 49, 423–429.
[15] Kahle, P., Hoppe-Seyler, O. and Kröger, H. (1971) Biochim. Biophys. Acta 240, 384–391.
[16] Kumar, A. (1969) Biochim. Biophys. Acta 186, 326–331.
[17] Macon, J. B. and Wolfenden, R. (1968) Biochemistry 7, 3453–3458.
[18] Baskin, F. and Dekker, C. A. (1967) J. Biol. Chem. 242, 5447–5449.
[19] Hindley, J. and Page, S. M. (1972) FEBS Lett. 26, 157–160.
[20] Faumann, M., Rabinowitz, M. and Getz, G. S. (1969) Biochim. Biophys. Acta 182, 355–360.