REACTION OF METHOXYAMINE WITH PHAGE f2 RNA AND ACTIVITY OF MODIFIED MESSENGER

W. FILIPOWICZ, A. WODNAR and P. SZAFRAŃSKI
Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw 12, Poland

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1. Introduction

Mode of action of hydroxylamine and methoxyamine on cytosine ring is well established [1–4]. Methoxyamine, in contrast to hydroxylamine, is not reactive towards uracil [5] and therefore can be used as a selective reagent in studies on nucleic acid structure and function. Cashmore et al. [6] have recently shown that in the case of *E. coli* tRNA, methoxyamine reacts mainly with sterically exposed cytosines which represent only about 20% of total cytosine content.

This work shows that methoxyamine can serve for structural and functional studies of natural messenger such as phage f2 RNA. The kinetics of reaction of methoxyamine with f2 RNA was studied using ^{14}C-labelled reagent. The results of melting and sedimentation analyses of methoxyamine-modified f2 RNA indicate that under conditions employed, methoxyamine treatment does not change the higher structure of f2 RNA molecules. However, modified f2 RNA alters significantly its capacity to code for phage specific polypeptides.

2, Materials and methods

O-methylhydroxylamine (methoxyamine) was purchased from Eastman Organic Chemicals; its solution was brought to pH 5.5 with sodium hydroxide. *O*-^{14}C-methylhydroxylamine was a product of New England Nuclear. All other chemicals used were as described previously [7].

Growth of *E. coli* Q13 cells, preparation of phage f2 and its RNA were as described in a previous paper [7]. f2 RNA used throughout the experiments contained 70% of the intact 29 S material as judged by analytical ultracentrifugation. To obtain methoxyamine-modified RNA, reaction mixtures containing 1 mg of f2 RNA in 0.3 ml of 1.0 M methoxyamine pH 5.5 and 10 mM Mg acetate were incubated in stoppered tubes for time and at temperature indicated in the figures. In control experiments reaction mixtures contained 1.0 M NaCl buffered with 10 mM phosphate pH 5.5 instead of methoxyamine. RNA was recovered by two precipitations with ethanol–acetate pH 5.5, washed once more with ethanol and dissolved in 1 mM EDTA pH 7.0. This RNA was used for incorporation experiments. For melting profiles determination, RNA solutions were additionally dialysed for 12 hr against 1 mM EDTA pH 7.0 and 12 hr against 0.1 mM EDTA pH 7.0.

Preparation of 30 S fraction from *E. coli* Q13 cells, incorporation of radioactive amino acids in the presence of f2 RNA, and polyacrylamide gel electrophoresis of ^{3}H-histidine and ^{14}C-alanine doubly-labelled products were as described previously [7] except that gel slices were solubilized and counted according to method of Matzura and Goodman [8] with the use of Beckman BBS-3 solubilizer.

Melting curves were determined in Unicam SP 500 spectrophotometer equipped with thermostated cuvette compartment. Sedimentation analysis of f2 RNA was carried out in 0.2 M NaCl, 0.005 M Tris-HCl pH 7.2 using Spinco Model E analytical centrifuge.

Other details are given in the text and figures.

3. Results and discussion

Fig. 1 shows that under conditions described the

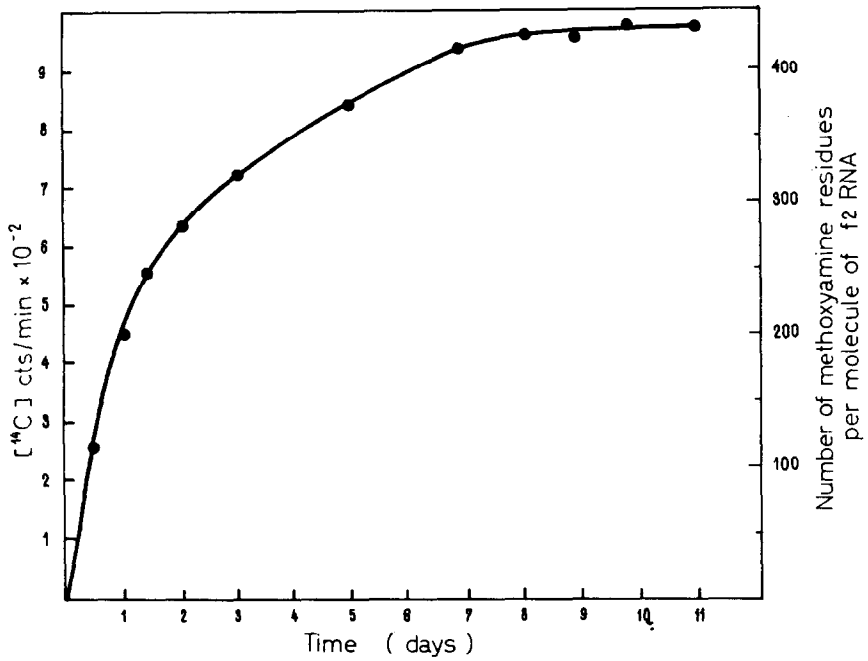

Fig. 1. Rate of reaction of ^{14}C-methoxyamine with f2 RNA. Reaction mixture contained: 3.3 mg of f2 RNA and 20 μCi of ^{14}C-methoxyamine in 1 ml of 0.1 M methoxyamine pH 5.5 and 10 mM Mg acetate. Incubation was carried out in a stoppered tube at 37°. At appropriate times 25 μl samples in duplicate were withdrawn, diluted 10 times with non-radioactive 1.0 M methoxyamine pH 5.5 and precipitated immediately with 1 vol of cold 10% TCA containing 1.0 M NH_4Cl. Precipitated RNA was kept in cold for 3 min, filtered through fiber glass FG 83 filters and washed 6 times with 5 ml cold 5% TCA containing 1.0 M NH_4Cl. Filters were subsequently transferred to the beaker containing 5% TCA with 1.0 M NH_4Cl, then washed once with ethanol–ether, once with ether, dried and counted in 10 ml of toluene scintillation mixture in the Packard Tricarb liquid scintillation counter. The value of 85 cpm corresponding to 0 hr of reaction has been subtracted. To calculate an uptake of methoxyamine groups per molecule of RNA a molecular weight 1.1×10^6 [9] was taken for the latter. Efficiency of isotope counting under conditions employed was estimated with the use of ^{14}C-uracil labelled f2 RNA of known specific activity.

reaction of methoxyamine with f2 RNA measured by ^{14}C-methoxyamine uptake proceeds relatively fast for 36 hr, then its rate lowers considerably and reaction stops after 5–7 days. That this is not due to the reagent decomposition was shown by the addition of fresh f2 RNA into the reaction mixture after 10 days of incubation. Newly added f2 RNA reacted with methoxyamine with the efficiency of about 75% of that observed at the beginning of the reaction. It was calculated that after 7 days of reaction about 400 methoxyamine groups reacted with one molecule of f2 RNA.

Reaction of methoxyamine with cytosine rings gives two different products: *N*-4-methoxycytosine (I) and 6-methoxyamino-*N*-4-methoxy-5,6-dihydrocytosine (II) [1,3]. Under conditions applied about half of modified cytosines present in methoxyamine-treated f2 RNA can be expected to be of type II [3, 6]. The value of 400 methoxyamine groups corresponds then to about 270 cytosine residues being methoxyaminated per RNA molecule. They represent about 30% of total 880 cytosines present in f2 RNA [9, 10]. A change in the reaction rate observed after about 36 hr of incubation (fig. 1) can indicate that reactive cytosines are not equally accessible for the reagent. The remaining 70% of cytosines do not react with methoxyamine even after prolonged treatment.

It was shown recently that in *E. coli* tRNA only those cytosines which are not engaged in the RNA secondary and tertiary structure are reactive with methoxyamine [6]. On the other hand the helical fraction in the RNA of small bacteriophages is estimated to be about 70% [11, 12]. As this number agrees

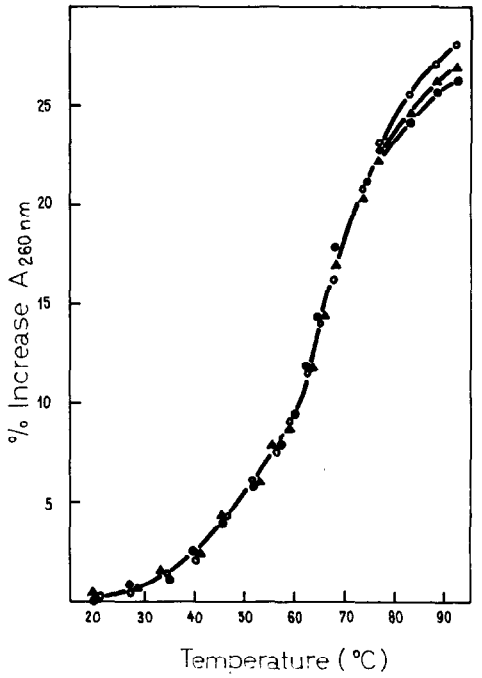

Fig. 2. Melting temperature profiles of methoxyamine-modified f2 RNA determined in SSC (0.15 M NaCl, 0.015 M Na citrate). RNA was treated with 1.0 M methoxyamine at 37° for 36 hr (●—●—●) and for 5 days (▲—▲—▲); control f2 RNA was incubated in 1.0 M NaCl for 36 hr at 37° (○—○—○). Details of RNA treatment and preparation in Methods.

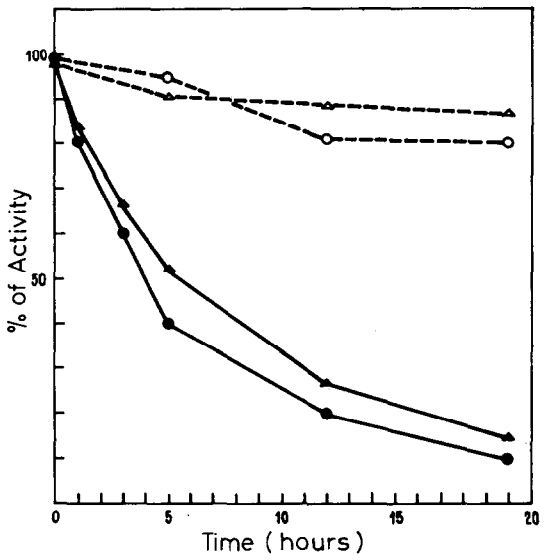

Fig. 3. Messenger activity of methoxyamine-modified f2 RNA as measured by incorporation of radioactive amino acids in *E. coli* cell-free system. Treatment of f2 RNA with methoxyamine as well as incubation of control RNA in NaCl solution was carried out at 21° for times indicated (for details see Methods). Methoxyamine-modified (▲—▲—▲) and control (△—△—△) f2 RNA directed ^{14}C-alanine incorporation; methoxyamine-modified (●—●—●) and control (○—○—○) f2 RNA directed ^{3}H-histidine incorporation. Activity of f2 RNA treated with methoxyamine for 0 hr was taken as 100%.

with the number of non-reactive cytosines found in our experiments it can be suggested that those are cytosines involved in the higher structure of f2 RNA.

That methoxyamine treatment does not change the higher structure of RNA was proved by comparison of melting and sedimentation properties of modified and unmodified f2 RNA. From fig. 2 it is evident that both have essentially the same temperature profiles. The results of analytical centrifugation showed that RNA treated with methoxyamine for 36 hr at 37° still contained 50% of material sedimenting with $s_{20,w}$ = 29.0 S; untreated RNA had the same sedimentation constant.

Results of studies on messenger activity of methoxyamine-treated f2 RNA in *E. coli* cell-free system are presented in fig. 3 and fig. 4. RNA used in these experiments was modified with methoxyamine at 21°. It can be seen from fig. 3 that the ability of modified RNA to stimulate the incorporation of alanine and histidine decreases with increasing time of f2 RNA treatment with methoxyamine. Histidine incorporation is slightly more impaired than that of alanine. The control RNA samples incubated parallelly do not show significant loss of stimulating activity. Both control RNA and RNA treated with methoxyamine for 20 hr were found to contain as much of intact 29 S material as starting RNA.

Polyacrylamide gel electrophoresis of ^{14}C-alanine and ^{3}H-histidine labelled polypeptides coded by control f2 RNA treated with methoxyamine for 0 hr reveals the synthesis of RNA-polymerase and coat protein as is observed with untreated RNA or RNA kept in 1.0 M NaCl for 12 hr (fig. 4A). Histidine counts present in the coat protein region can correspond to some unfinished or degraded polymerase polypeptides. f2 RNA treated with methoxyamine for 12 hr loses its ability to synthesize complete RNA-polymerase, while the coat protein formation is much less affected

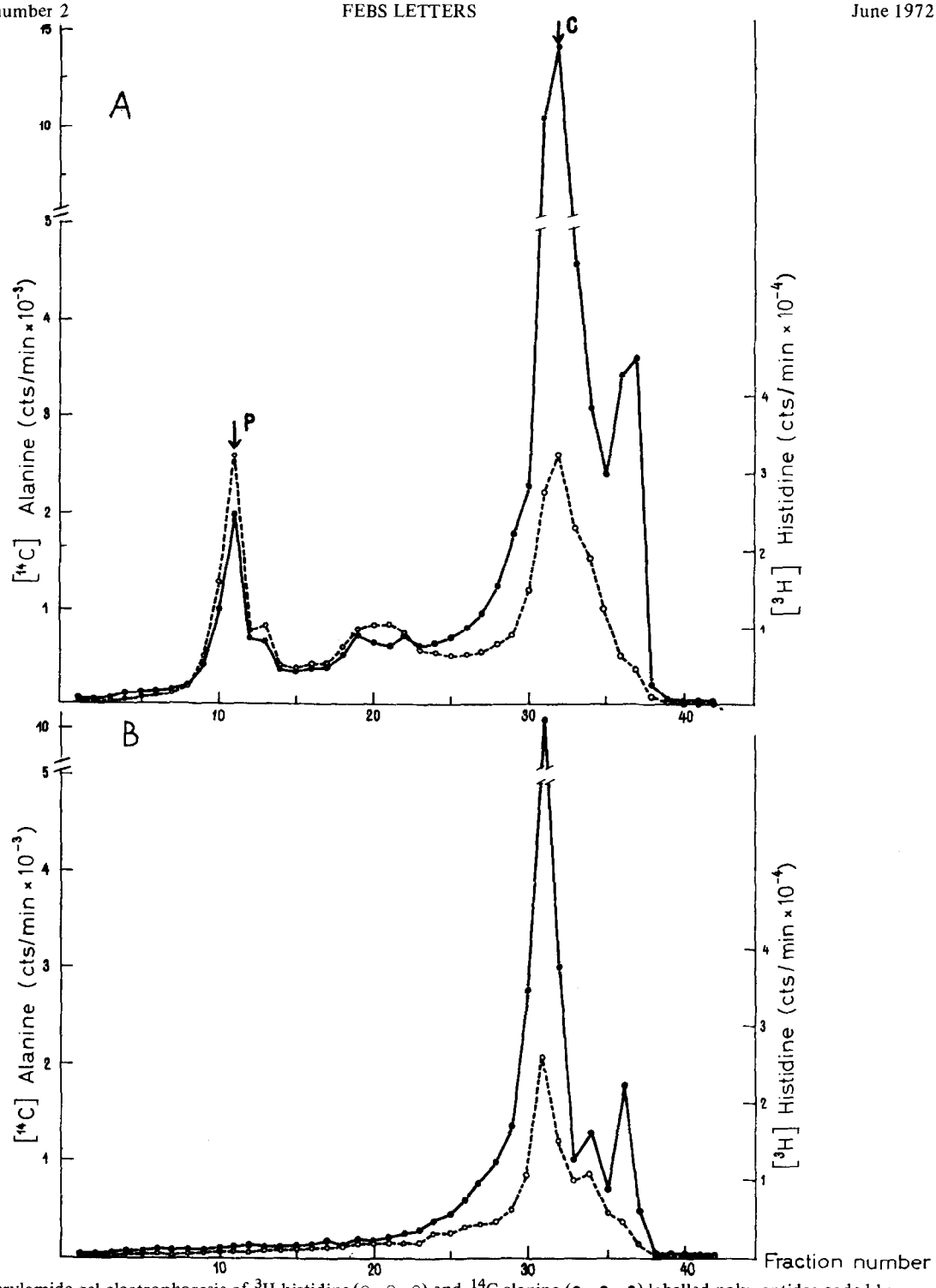

Fig. 4. Polyacrylamide gel electrophoresis of ^{3}H-histidine (○---○---○) and ^{14}C-alanine (●—●—●) labelled polypeptides coded by methoxyamine-modified f2 RNA. RNA used for incorporation of amino acids [7] was treated with 1.0 M methoxyamine pH 5.5 containing 10 mM Mg acetate, at 21° for: 0 hr (A) and 12 hr (B). Details for DNA preparation in Methods. Arrows refer to the positions of RNA-polymerase (P) and coat protein (C).

(fig. 4B). It can be suggested that modified cytosines distributed along f2 RNA chain block the elongation of growing polypeptides.

In view of these results it seems that methoxyamine, reacting only with non base-paired cytosines, is a promising tool to study which fragments are exposed in f2 RNA structure and whether single stranded and/or double stranded regions of f2 RNA are involved in its interactions with ribosomes or coat protein acting as a repressor.

Acknowledgements

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