Two transfer RNA (1-methylguanine) methylases from yeast

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Received 5 May 1975

ABSTRACT
Two distinct tRNA (m^1G) methylases have been found in the yeast Saccharomyces cerevisiae. They differ in their chromatographic properties on hydroxyapatite, in their response to spermine, and in their site specificity. Only one of the methylases is active against normal tRNA from Escherichia coli.

INTRODUCTION
Most tRNA's examined so far contain methylated nucleosides. The methylation occurs at the polynucleotide level and is effected by a set of highly specific enzymes, tRNA methylases. Although most of the methylated nucleosides in tRNA are probably known today, only a few of the corresponding methylases have been demonstrated and described.

Hurwitz et al. (1) have reported two methylating activities in E. coli, both producing m^1G in tRNA. Since they exhibit the same site specificity, they probably represent the same enzyme. The presence of a tRNA (m^1G) methylase in E. coli has been further confirmed (2,3).

The presence of tRNA (m^1G) methylase in yeast was first reported by Björk and Svensson (4). In this paper, we present evidence for two different enzymes in yeast producing m^1G.

MATERIALS AND METHODS
Strains and growth conditions
Saccharomyces cerevisiae, strain D38, and Escherichia coli, strains B and K12 58-161 (known as W6), were grown as described earlier (4). Strain D38 is a mutant lacking an active tRNA (m^2G) methylase (5). Strain W6 is met^- and RCre^rel, and it produces a
generally submethylated tRNA during methionine starvation.

Other materials

S-Adenosyl-L-(methyl-\(^{14}\)C)-methionine (54.6 mCi/mmole) was obtained from New England Nuclear Corp. DNase I, spermine, and protective lacquer spray were purchased from Sigma Chemical Co. Sheets No 6064 used for thin-layer chromatography were bought from Eastman Kodak Co. Hydroxyapatite was prepared according to Tiselius et al. (6).

Preparation of tRNA

Transfer RNA was prepared as previously described (7).

Preparation of yeast extract

A crude enzyme extract from S. cerevisiae D38 was prepared similarly to Björk and Svensson (4). To 30 ml of the extract was added 500 mg of streptomycin, dissolved in 10 ml of 50 mM potassium phosphate buffer, pH 6.8, containing 5 mM 2-mercaptoethanol. After stirring for 1 h at 0°C, the precipitate was removed by centrifugation for 15 min at 20,000 x g. To the supernatant was added solid ammonium sulphate to give 70% saturation. After standing for 1 h at 0°C, the precipitate was collected by centrifugation for 15 min at 20,000 x g and suspended in 5 ml of the same buffer (AS-fraction).

Assay for methylase activity

For routine methylase assay of chromatograms, the reaction mixture contained, in a total volume of 0.2 ml: 0.1 M Tris-HCl, pH 8.0, 1 mM dithiothreitol, 0.1 mM EDTA, 10 mM MgSO\(_4\), 20 mM NH\(_4\)Cl, 0.45 nmoles of \(^{14}\)C-labelled SAM, 50 \(\mu\)g of tRNA, and 0.1 ml of the enzyme fraction. Incubation was at 30°C for 1 h. The samples were then treated and measured as previously described (8).

The efficiency of liquid scintillation counting of glass filters was 75%.

Assay of tRNA (m\(^{15}\)G) methylase was performed in the same way, followed by analysis of methylated bases.

Characterization of methylated products

To each sample of methylated tRNA was added 0.2 mg of carrier tRNA. After recovery of tRNA from the incubation mixtures by
phenol extraction, the methylated constituents were analyzed as already described (4,9).

The efficiency of liquid scintillation counting of thin-layer sheets, sprayed with protective lacquer, was 45%.

RESULTS
Fractionation of yeast tRNA methylases

The freshly prepared AS-fraction from S. cerevisiae D38 was chromatographed on Sephadex G-150. As shown in Fig. 1, all tRNA-methylating activity was eluted in the first protein peak.

![Fig. 1. Chromatography of AS-fraction from yeast on Sephadex G-150. Column size was 1.4 cm x 90 cm. 50 mM potassium phosphate buffer, pH 6.8, containing 5 mM 2-mercaptoethanol, was used for both equilibration and elution. 6 ml of AS-fraction, containing 300 mg of protein, was applied on the column, and fractions of 2.0 ml were collected at a flow rate of 6 ml/h. The temperature was kept at 4°C. (-----), absorbance at 254 nm; (o——o), methyl group incorporation into submethylated E. coli tRNA, expressed as uncorrected cpm. The active fractions were pooled as indicated.

The active fractions were collected and further fractionated on a hydroxyapatite column, using a linear phosphate gradient. The distribution of methylases active against normal and submethylated E. coli tRNA is shown in Fig. 2. Base analysis of the methylated tRNA has revealed two activities producing \textsuperscript{m}G in
submethylated tRNA. One elutes at 0.08 - 0.09 M phosphate (fraction I) and is active against both normal and submethylated tRNA. The other activity (fraction II) elutes at 0.260 - 0.275 M phosphate and is active against submethylated tRNA but not against normal tRNA.

Fig. 2. Chromatography on hydroxyapatite of the pooled active fractions from Sephadex G-150. Column size was 1.4 cm x 30 cm. The column was equilibrated with 50 mM potassium phosphate buffer, pH 6.8, containing 5 mM 2-mercaptoethanol. The pooled material (50 ml with 150 mg of protein) was applied, and fractionation was achieved by a linear gradient of 0.05 - 0.45 M of the same phosphate buffer (250 ml of each) at a flow rate of 6.0 ml/h. 2.0 ml fractions were collected. The temperature was kept at 4°C. (-----), absorbance at 254 nm; (○-○), standard assay for methylase activity against submethylated E. coli tRNA; (●-●), standard assay of methylase activity against normal E. coli tRNA; (□-□), phosphate buffer concentration. Values in cpm are uncorrected. The fractions containing tRNA (m1G) methylase activity were pooled as indicated.
The fractions I and II, containing tRNA \((m^1G)\) methylase, were pooled as shown in Fig. 2.

Product analysis

The products obtained after methylation of E. coli tRNA for 8 h with fractions I and II were analyzed (Table I). Fraction I contains almost only methylases for \(m^1G\) and \(m^5C\), while fraction II contains mainly methylases for \(m^1G\) and \(m^5U\). Except for small amounts of unknown methylated products, possibly representing ribose-methylated nucleosides, no other methylated compounds were found.

Analysis of products after 1 h incubation shows that tRNA \((m^1G)\) methylase corresponds to about 20% of the total methylating activity against submethylated E. coli tRNA for both enzyme fractions.

Table I. Pattern of methylation of E. coli tRNA by fractions I and II. Incubation with standard reaction mixture was for 8 h, followed by base analysis. No radioactivity was detected in \(m^2G\), \(m^3G\), \(m^4G\), \(m^4A\), \(m^5A\) and \(m^6A\). The unknown compounds possibly represent ribose-methylated nucleosides. The tRNA from strain W6 was submethylated.

<table>
<thead>
<tr>
<th>Enzyme fraction</th>
<th>tRNA source</th>
<th>Cpm recovered from thin-layer</th>
<th>Recovery (%)</th>
<th>Recovered radioactivity in % of total recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(m^1G)</td>
</tr>
<tr>
<td>I</td>
<td>W6</td>
<td>3098</td>
<td>96</td>
<td>21</td>
</tr>
<tr>
<td>I</td>
<td>B</td>
<td>2233</td>
<td>102</td>
<td>5</td>
</tr>
<tr>
<td>II</td>
<td>W6</td>
<td>2094</td>
<td>94</td>
<td>20</td>
</tr>
</tbody>
</table>

Stability of the methylase fractions

Fraction I can be stored for several days at 4°C without any apparent loss of activity, while fraction II which contains much less protein loses its activity rapidly (Fig. 3). The experiments to be reported were therefore always performed with fresh enzyme.
Fig. 3. Stability of fractions I and II at 4°C. 100% of methylating activity represents an initial rate of methylation of 7600 dpm/h for fraction I (○—○) and 6470 dpm/h for fraction II (●—●). Standard assay with submethylated E. coli tRNA was employed.

**Temperature sensitivity**

Fractions I and II also differ in temperature sensitivity. Table II shows that preincubation for 1 min at 45°C almost completely destroys the activity of fraction II, while most of the activity of fraction I remains. Preincubation for 1 min at 60°C abolishes the methylating activity of both fractions.

Table II. Temperature sensitivity of fractions I and II. Prior to standard assay with submethylated E. coli tRNA, the enzyme fractions were preincubated for 1 min at the temperatures given. Values are given in % of control without preincubation. 100% of enzyme activity represents 10 267 dpm/h for fraction I and 4 267 dpm/h for fraction II.

<table>
<thead>
<tr>
<th>Enzyme fraction</th>
<th>Preincubation temperature</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>30°C</td>
</tr>
<tr>
<td>I</td>
<td>100</td>
</tr>
<tr>
<td>II</td>
<td>100</td>
</tr>
</tbody>
</table>
Response to magnesium ions

The effect of Mg$^{2+}$ on the rate of m$^1$G formation in submethylated E. coli tRNA with the two methylase fractions was studied. Standard assay conditions, except for EDTA and NH$_4^+$, was used, followed by base analysis of the methylated products. Both enzymes were active in the absence of Mg$^{2+}$, but the addition of Mg$^{2+}$ in the range 2 - 5 mM resulted in an increase of reaction rate by several hundred per cent. The differences found between the two enzymes were not significant.

Influence of spermine

The rate of reaction of both tRNA (m$^1$G) methylases was greatly increased by addition of spermine to a standard reaction mixture, from which Mg$^{2+}$ and NH$_4^+$ were omitted. As shown in Fig. 4, the enzyme in fraction I exhibits an optimum at 1 mM spermine or higher, while the enzyme in fraction II has its main optimum around 0.5 mM spermine and possibly a second optimum around 3 mM. Although the data are somewhat uncertain, this tendency was found in three independent experiments. A similar phenomenon with two optima for spermidine was observed by Hacker (10).

![Fig. 4. Influence of spermine on tRNA (m$^1$G) methylase I and II. Standard assay with submethylated E. coli tRNA, omitting Mg$^{2+}$ and NH$_4^+$, was used, followed by analysis of methylated bases. 100% of enzyme activity represents a recovery of 361 dpm in m$^1$G for enzyme I (○–○) and 220 dpm for enzyme II (●–●). The data given are mean values from three independent experiments.](image)
Specificity of the tRNA (m\(^1\)G) methylases

The chromatographic pattern and the properties of the two tRNA (m\(^1\)G) methylases indicate that the two activities represent different enzymes. We have therefore attempted to characterize them further with emphasis on their site specificities. Table III shows the apparent extent of m\(^1\)G formation with the two enzymes in both normal and submethylated E. coli tRNA. The data given for enzyme I do probably not represent maximum formation of m\(^1\)G. For enzyme II, however, we have been able to ascertain that addition of more enzyme does not increase the incorporation value.

Table III. Extent of tRNA methylation with tRNA (m\(^1\)G) methylases I and II. Incubation was carried out for 24 h at 30°C in standard assay mixture with addition of 1 mM spermine, followed by base analysis.

<table>
<thead>
<tr>
<th>Enzyme fraction</th>
<th>Submethylated E. coli tRNA</th>
<th>Normal E. coli tRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dpm recovered in m(^1)G</td>
<td>mmoles of m(^1)G per mole of tRNA</td>
</tr>
<tr>
<td>I</td>
<td>1130</td>
<td>65</td>
</tr>
<tr>
<td>II</td>
<td>200</td>
<td>18</td>
</tr>
</tbody>
</table>

It is evident from Table III that enzyme I but not enzyme II can recognize normal E. coli tRNA, which confirms the data in Fig. 2. Both enzymes find sites for m\(^1\)G formation in submethylated tRNA, but enzyme I obviously recognizes sites which enzyme II cannot. This is further substantiated in Fig. 5. After reaching plateau in m\(^1\)G synthesis with enzyme II, addition of more enzyme II does not increase the plateau value, while addition of enzyme I significantly does. We therefore conclude that the two tRNA (m\(^1\)G) methylases found in yeast are different enzymes with different site specificities.

DISCUSSION

The apparent activity of the two tRNA (m\(^1\)G) methylases from yeast is low, which has been a hindrance to obtain reliable kinetic data. We have tried to estimate the difference between the rates of m\(^1\)G formation in tRNA in vitro and in vivo. The
Fig. 5. Extent of methylation by tRNA (m\textsuperscript{1}G) methylases I and II. Standard methylation mixture was used, containing 7.2 \(\mu\)g of sub-methylated E. coli tRNA and 1 mM spermine in 200 \(\mu\)l. Methylase II (50 \(\mu\)g of protein per ml of reaction mixture) was first added (o—o). After 8 h of incubation, 100 \(\mu\)l of methylase I containing 200 \(\mu\)g of protein (●—●) or 100 \(\mu\)l of methylase II (o—o) was added to parallel series of tubes. Base analysis was performed as described.

Our calculations indicate that the turnover number of tRNA (m\textsuperscript{1}G) methylase I is roughly 100 times higher in vivo than in vitro. Since tRNA is probably rate-limiting in vivo, the difference found must be regarded as a minimum value.

We have found a similar disproportion between rates of methylation in vitro and in vivo for other tRNA methylases from both yeast and E. coli. Calculations based on data from Davis and Nierlich (3) concerning E. coli tRNA methylases also give similar results. Thus, it is not probable that the slow rate of
reaction with our yeast enzyme depends on the heterologous substrate tRNA.

There are two sites for m^1G known in yeast tRNA, one at position 9 and one at position 43 next to the anticodon (11). No other sites for m^1G have been established for any tRNA from any organism so far. About 20 different tRNA's from yeast have been sequenced. Nine of these have m^1G at position 9 and two at position 43. It is a generally accepted view that the tRNA methylases have a pronounced site specificity. One would therefore expect two different tRNA (m^1G) methylases in yeast, one with specificity for site 9 and the other for site 43. Our data conform to this model as far as that we have found two enzymes with different site specificities. It is not probable that the use of heterologous tRNA would confer a change in site specificity to the enzymes. Two different methylases for the same methylated base at two different positions has been demonstrated by Kraus and Staehelin (12,13) for m^2G formation in rat liver tRNA.

It has been reported that E. coli tRNA contains about 0.14 moles of m^1G per mole of tRNA (14). This quantity implies that about ten of the specific tRNA species contain m^1G. By now, over 30 tRNA's from E. coli have been sequenced, but only one tRNA (Leu 1) is suggested to contain m^1G, at position 43 (15). However, Blank and Söll (16) have questioned this and claim acG at this position. Two chromatographically distinct tRNA (m^1G) methylases have been isolated from E. coli (1), but since they obviously attack the same site in tRNA, they probably represent different forms of the same enzyme. Similar situations have been reported by other investigators (17,18).

Supposing that the E. coli tRNA (m^1G) methylase recognizes guanine at position 43, normal E. coli tRNA should be fully methylated at this site and should not accept methyl groups from
the corresponding yeast methylase. Our data show that yeast tRNA (m'G) methylase II does not methylate normal E. coli tRNA. This enzyme might therefore be specific for guanine at position 43. The low maximum incorporation value found is equivalent to one methyl group in two species of tRNA.

The other yeast enzyme, tRNA (m'G) methylase I, may have its specificity directed towards position 9. Several normal tRNA's from E. coli possess an unmodified guanine at this position and are a priori candidates as substrates for the yeast methylase. Our results show that yeast tRNA (m'G) methylase I readily methylates normal E. coli tRNA. The highest incorporation value attained corresponds to one methyl group in two species of tRNA.

If our model for the site specificities of the two methylases is true, we would expect the same maximum incorporation in both normal and submethylated E. coli tRNA by yeast tRNA (m'G) methylase I. This has not come out true in our experiments. We have reasons to believe that our data concerning the extents of methyl group incorporation are too low. Work is in progress to settle this problem, using purified tRNA's with known sequences.

ACKNOWLEDGEMENTS

The work has been supported by The Ollie and Elof Ericsson Fund, and The Knut and Alice Wallenberg Fund.

REFERENCES