Comparative structural analysis of eubacterial 5S rRNA by oxidation of adenines in the N-1 position

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ABSTRACT

Adenines in free 5S rRNA from Escherichia coli, Bacillus stearothermophilus and Thermus thermophilus have been oxidized at their N-1 position using monoperphthalic acid. The determination of the number of adenine 1-N-oxides was on the basis of UV spectroscopic data of the intact molecule. Identification of the most readily accessible nucleotides by sequencing gel analysis reveals that they are located in conserved positions within loops, exposed hairpin loops and single-base bulge loops. Implications for the structure and function of 5S rRNA will be discussed on the basis of this comparative analysis.

INTRODUCTION

5S ribosomal RNA is an integral, functionally essential constituent of the eubacterial as well as of the eukaryotic ribosome. Comparative structural analysis has led to a general secondary structure for eubacterial 5S rRNA (1-6), which is in agreement with a large number of physical, chemical and biochemical studies (reviewed in 7 and 8). However, its tertiary structure as well as its functional properties remain to be worked out.

Base specific, partial chemical modification has been used in order to gain information about the tertiary structure of the 5S rRNA molecule (9,10,19). We have focused our interest on adenines since they have been implicated as particularly important for the structure and function of the molecule. Indeed the majority of the adenines are located in single stranded rather than in double stranded parts of the molecule, and herein many of them are conserved. Moreover, they are involved in the formation of single or double base bulge loops, which have been implied to be important for protein recognition and/or interaction (4,9).
In this study we will describe the modification of adenines at the N-1 position using monoperphthalic acid. This reaction has been described for E. coli 5S rRNA in situ, in the 50S ribosomal subunit, demonstrating the accessibility of A₁, and A₂, (10). Up to ten adenines can be oxidized in free 5S rRNA from E. coli (11). We have identified the fastest reacting adenines in free 5S rRNA from three eubacteria: E. coli, B. stearothermophilus and T. thermophilus. In addition, we will describe a method which allows the exact determination of the number of modified adenines solely from UV spectroscopic data on the intact molecule.

MATERIALS AND METHODS
Isolation of 5S rRNA
E. coli (MRE 600) and B. stearothermophilus (799) 5S rRNAs were purified from 70S ribosomes as described (12). 5S rRNA from T. thermophilus (HB 8), a generous gift from Dr. I. Kumagai, Tokyo, was isolated as published (12,13).

Chemical modification
Oxidation of adenines at the N-1 position was performed principally as reported (10,11,14 and 15) with minor modifications. Monoperphthalic acid (MPPA) was prepared as described by Payne (16). In a standard assay, 100 μg (≈ 2.5 nmol) of 5S rRNA were incubated in the dark with 9.2 μM MPPA in 75 μl 0.4 M phosphate buffer, pH 7.0 at room temperature for 0.25 to 8 hours.

The 5S rRNAs, which were used for the identification of modified nucleotides, were incubated for 2 hours. The reaction was stopped by gel chromatographic separation of RNA and MPPA on a Sephadex G-50 column (1.5 x 15.0 cm) in H₂O. The peak fractions containing RNA were pooled and analyzed UV spectroscopically in a Beckman DU 8 spectrophotometer. Finally, the RNA was precipitated by the addition of two volumes ethanol and 0.1 volume 1M sodium acetate, pH 5.0 at -20°C overnight. For the subsequent identification of modified nucleotides by sequencing gel analysis, the RNA was redissolved in H₂O to a concentration of 0.5 mg/ml.

Identification of modified nucleotides
5S rRNA was labelled at the 3'end and sequenced using the en-
zymatic degradation procedure described by Donis-Keller et al. (17).

RESULTS

Estimation of the extent of adenine modification

The UV spectra for native and modified E. coli 5S rRNA are shown in Figure 1. An additional peak arises in the N-oxidized fraction at 232nm, which is due to a maximum in the absorption profile of AMP-1-N oxide at this wavelength.

Thus, the quotient $Q = \frac{A_{232}}{A_{260}}$ is proportional to the extent of chemical modification. The kinetics of the reaction are shown in Figure 2. These data are in agreement with the previous results from Cramer and Erdmann (11). Given the individual extinction coefficients, which are listed in Table I, it is possible to calculate the number of AMP-1-N-oxides from $Q$:

$$E_{232} = \varepsilon_G \cdot c_G \cdot d + \varepsilon_C \cdot c_C \cdot d + \varepsilon_U \cdot c_U \cdot d + \varepsilon_A \cdot c_A \cdot d + \varepsilon_X \cdot c_X \cdot d$$

$$E_{260} = \varepsilon_G \cdot c_G \cdot d + \varepsilon_C \cdot c_C \cdot d + \varepsilon_U \cdot c_U \cdot d + \varepsilon_A \cdot c_A \cdot d + \varepsilon_A \cdot c_A \cdot d$$

defining $C \equiv N$, the number of each nucleotide in a given molecule and $d \equiv 1\text{cm}$, we can calculate for E. coli with

![Figure 1. UV spectra of E. coli 5S rRNA (A), and E. coli 5S rRNA reacted with monoperphthalic acid under standard conditions (see Materials and Methods) for 8 hrs. (B).](image-url)
Figure 2. Time course of *E. coli* 5S rRNA N-oxidation with monoperphthalic acid. Other details of reaction conditions are given under Materials and Methods.

\[ N_G = 42 \]
\[ N_C = 35 \]
\[ N_U = 20 \]

\[ E_{232} = 520 \cdot 10^3 + N_A \cdot 3.2 \cdot 10^3 + N^* \cdot 40.6 \cdot 10^3 \]
\[ E_{260} = 944 \cdot 10^3 + N_A \cdot 15.3 \cdot 10^3 + N^* \cdot 7.7 \cdot 10^3 \]

Elimination of either *N*\(_A\) or *N*\(^\ast\) results in:

\[ E_{232} - 5.17 E_{260} = -4360 \cdot 10^3 - 75.9 \cdot 10^3 \cdot N_A \]
\[ E_{260} - 4.78 E_{232} = -1542 \cdot 10^3 - 183.0 \cdot 10^3 \cdot N^*_A \]

Replacement of the absolute values for *E*\(_{232}\) and *E*\(_{260}\) by the relative factor \( Q = \frac{E_{232}}{E_{260}} \), which is determined experimentally, leads to:

\[ Q - 5.17 = (-4360 \cdot 10^3 - 75.9 \cdot 10^3 \cdot N_A) \frac{1}{E_{260}} \]
\[ 1 - 4.78 Q = (-1542 \cdot 10^3 - 183 \cdot 10^3 \cdot N^*_A) \frac{1}{E_{260}} \]

and

\[ \frac{Q - 5.17}{-4360 - 75.9 N_A} = \frac{1 - 4.78 Q}{-1542 - 183 N^*_A} \]

With \( N_A + N^*_A = NT_A = 23 \), which is the total number of adenines in *E. coli* 5S rRNA, it is now possible to calculate the number of modified adenines for any experimental value of \( Q \). The results are shown in Figure 3, in comparison to the data obtained.
Table I
Extinction coefficients for ribonucleoside monophosphates at pH 7.0.

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>$\lambda$ [nm]</th>
<th>$\varepsilon$ [mol$^{-1}$ cm$^{-1}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>$2'$($3'$)-GMP</td>
<td>232$^a$</td>
<td>4.9 $\cdot$ 10$^3$</td>
</tr>
<tr>
<td>$2'$($3'$)-CMP</td>
<td>232$^a$</td>
<td>7.7 $\cdot$ 10$^3$</td>
</tr>
<tr>
<td>$2'$($3'$)-UMP</td>
<td>232$^a$</td>
<td>2.1 $\cdot$ 10$^3$</td>
</tr>
<tr>
<td>$2'$($3'$)-AMP</td>
<td>232$^a$</td>
<td>3.2 $\cdot$ 10$^3$</td>
</tr>
<tr>
<td>$2'$($3'$)-AMP-1-N-oxide</td>
<td>232$^b$</td>
<td>40.6 $\cdot$ 10$^3$</td>
</tr>
<tr>
<td>$2'$($3'$)-GMP</td>
<td>260$^a$</td>
<td>11.7 $\cdot$ 10$^3$</td>
</tr>
<tr>
<td>$2'$($3'$)-CMP</td>
<td>260$^a$</td>
<td>7.3 $\cdot$ 10$^3$</td>
</tr>
<tr>
<td>$2'$($3'$)-UMP</td>
<td>260$^a$</td>
<td>9.9 $\cdot$ 10$^3$</td>
</tr>
<tr>
<td>$2'$($3'$)-AMP</td>
<td>260$^a$</td>
<td>15.3 $\cdot$ 10$^3$</td>
</tr>
<tr>
<td>$2'$($3'$)-AMP-1-N-oxide</td>
<td>260$^b$</td>
<td>8.0 $\cdot$ 10$^3$</td>
</tr>
</tbody>
</table>

$^a$Data taken from Pabst Laboratories, Milwaukee, Wisconsin, Circular OR 10.

$^b$Data taken from Cramer and Schlingloff (18).

The number of AMP-1-N-oxides as calculated from
$Q = \frac{E_{260}}{E_{232}}$, (x) and as determined after separation of the individual mononucleotides from the hydrolysate of E. coli 5S rRNA (o), data from ref. (11,14).
The number of AMP-1-N-oxides in three different eubacterial 5S rRNAs, calculated from $Q = \frac{E_{193}}{E_{260}}$ as discussed in the text. Modifications were carried out under standard conditions for 2 hrs.

<table>
<thead>
<tr>
<th>Organism</th>
<th>$Q$</th>
<th>AMP-1-N-oxides/5S rRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>0.703</td>
<td>7.5</td>
</tr>
<tr>
<td>B. steaothermophilus</td>
<td>0.777</td>
<td>10.1</td>
</tr>
<tr>
<td>T. thermophilus</td>
<td>0.773</td>
<td>9.4</td>
</tr>
</tbody>
</table>

mental curve yields values which are 5 to 10% smaller than the calculated ones. This difference may be due to structural effects such as stacking interactions in the native molecule.

Similar calculations have been performed for T. thermophilus and B. steaothermophilus 5S rRNA, and the results for those fractions, which have been used for the identification of modified adenine positions are summarized in Table II.

Identification of modified adenines

Examination of E. coli 5S rRNA modified in situ, in the 50S ribosomal subunit, revealed that the modified adenines in position 73 and 99 (10) are no longer recognized by the adenine specific ribonuclease U, and Phy M in the corresponding enzymatic sequencing reactions (17). This effect has been used for the identification of adenine-1-N-oxides produced in free E. coli, B. steaothermophilus and T. thermophilus 5S rRNA. The sequencing gel analysis is shown in Figure 4. A major reduction of the reactivity in both adenine specific reactions (U, and Phy M) has been observed for $A_{66}$, $A_{73}$, and $A_{83}$, in E. coli, for $A_{73}$, $A_{83}$, $A_{73}$, and $A_{83}$, in B. steaothermophilus and for $A_{73}$, $A_{83}$ and $A_{102}$, in T. thermophilus. Minor effects occurred at $A_{73}$, $A_{104}$ and $A_{108}$, in E. coli as well as at $A_{73}$, $A_{83}$, $A_{104}$, $A_{73}$, and $A_{102}$, in B. steaothermophilus and at $A_{102}$, in T. thermophilus. These results are summarized in Figure 5. In some cases, minor effects were also observed in the G reaction for $G_{73}$, in B. steaothermophilus as well as $G_{73}$, and $G_{83}$, in T. thermophilus.
Figure 4. Base specific, enzymatic hydrolysis of 5S rRNA from
A) E. coli, B) B. stearothermophilus and C) T. thermophilus.
Lane 1: Incubation of 3' labelled 5S rRNA in the absence of en-
zymes,
lane 2: alkali hydrolysis,
lane 3: T1 digestion (G specific) of 5S rRNA,
lane 4: T1 digestion after MPPA treatment,
lane 5: U2 digestion (A specific) of 5S rRNA,
lane 6: U2 digestion after MPPA treatment,
lane 7: Phy M digestion (A, U specific) of 5S rRNA,
lane 8: Phy M digestion after MPPA treatment.
Figure 5. Secondary structure of eubacterial 5S rRNAs. Squares indicate base paired and circles single stranded regions. Conserved and semiconserved positions (R = purine and Y = pyrimidine) are indicated. Arrows symbolize sites of primary chemical modification of adenosines by monoperphthalic acid in E. coli, B. stearothermophilus and T. thermophilus 5S rRNA.
DISCUSSION

Monoperphthalic acid has been shown to convert AMP to AMP-1-N-oxide under mild conditions (11,14,15). No reaction occurs if nitrogen 1 is protected by hydrogen bonding (15) and analysis of oxidizable adenines in yeast tRNA_{Phe} has shown that they are located in the exposed anticodon loop and the CCA terminus (15), which are two of the functionally significant regions in tRNA.

The kinetics of the *E. coli* 5S rRNA N-oxidation are shown in Figure 2. Up to 11 adenines can be modified, which is in agreement with earlier results (11).

The most readily accessible adenines in eubacterial 5S rRNA are located in conserved, single stranded positions (Figure 5). They are identical to those adenines which could be modified in situ, in the 50S ribosomal subunit of *E. coli* (10), indicating that they are not involved in the interaction with ribosomal proteins. They are, on the other hand, exposed and possibly involved in the interaction with other ribosomal RNAs as discussed elsewhere (10). The other adenines, located in loops A, B, C, D, E and G, which react poorly or not at all with monoperphthalic acid, are likely to be either buried within the tertiary structure of the molecule or involved in tertiary interactions. Such interactions have been postulated for loop C and loop E (4, 19-21), but these structures are only partly resolved.

A complication in the study of 5S rRNA structure has been the observation of structural heterogeneity in *E. coli*. The occurrence of structurally distinct A-form (native) and B-form (denatured) has been well documented (22,23). In addition, evidence is accumulating for a low-temperature melting transition in A-form 5S rRNA (24-26), which is a result of changes in temperature, pH and counterion concentration. Both conformational states (high- and low-temperature state) were shown to bind the ribosomal protein EL5 (26). Under the experimental conditions we have used for the chemical modification (low salt, absence of Mg^{++}) 5S rRNA exhibits a biphasic melting behaviour (11) which is typical for native 5S rRNA. The experimental data therefore refer to the low temperature state of native *E. coli* A-form 5S rRNA.

The formation of a fifth helix between loop E and G, similar
to the one found in eukaryotic 5S rRNAs, has been proposed for *E. coli* (27) and the other eubacterial 5S rRNAs (2,5,6) including "odd" base pairs such as A-G (6,27). Our experimental findings that A₇ and A₆₈ in *E. coli* and the corresponding adenines in the other eubacterial 5S rRNAs are most readily oxidizable in their N-1 position suggest that they are not involved in base pairing or other hydrogen bonding. These data support our earlier hypothesis on the existence of two functionally and structurally distinct classes of 5S rRNA, the eubacterial and the eukaryotic one (28).

The adenines present in loop F of *B. stearothermophilus* and *T. thermophilus* 5S rRNA were also found to react with monoperphthalic acid. Thus, this hairpin loop does not seem to be involved in tertiary interactions but, on the contrary, to be exposed, comparable to the anticodon loop in tRNA, which was also shown to be accessible in the same reaction (15).

Unexpected effects have been observed for the bulged adenine of helix II, comparing the three 5S rRNA species examined. A₆₆ in *E. coli* is clearly oxidizable, which is consistent with the data from diethylpyrocarbonate modification of purines in the N-7 position (9), where the same nucleotide was found to be the most reactive. The same authors have shown that A₆₆ is involved in binding of protein E-L18, which is again in agreement with our data, as this nucleotide is inaccessible *in situ* in the 50S ribosomal subunit of *E. coli* (10).

However, the corresponding adenine in *B. stearothermophilus* (A₆₄) was found to be significantly less reactive in the monoperphthalic acid reaction and A₆₈ in *T. thermophilus* did not exhibit any detectable reactivity. These data are in line with our results from comparative nuclease S₁ digestion studies (29) on the structural dynamics of 5S rRNA, where we have shown that only the bulged adenine of *E. coli* is accessible for this enzyme under a variety of conditions. These findings indicate different conformations for this particular bulged adenine in the three 5S rRNA species tested. We consider the possibility that bulged nucleotides may adopt either a 'looped out' conformation, protruding from the surrounding helical segments, or, alternatively, an 'intercalated' conformation, stacked between the neighbouring
base pairs. The equilibrium between these two conformations may well be influenced by the adjacent base pairs of the bulged nucleotide, explaining the relative hyperreactivity of $A_{6}$ in E. coli. Here, the bulged adenine is flanked by a G-C and a G-U basepair, whereas in the other two species the flanking basepairs are both G-C. The conserved double base bulge loop in helix III may also adopt an intercalated conformation, as these nucleotides did not react with monoperphthalic acid.

"Wedged" nucleotides might serve the purpose of bending adjoining helical segments to a fixed angle, resulting in a specific relative orientation of the corresponding structural domains.

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REFERENCES