Destabilization of secondary structure in 16S ribosomal RNA by dimethylation of two adjacent adenosines

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ABSTRACT

Fragments comprising the 49 nucleotides from the 3'-end have been purified from 16S ribosomal RNA of wild-type Escherichia coli and from a kasugamycin-resistant mutant that specifically lacks dimethylation of two adjacent adenosines near the 3'-terminus. These fragments, obtained after treatment of ribosomes in vitro with the bacteriocin cloacin DF13, were used to study the effect of the methyl groups on the temperature dependent unfolding of double-stranded regions. Both fragments contain at least 3 independent melting transitions, of which the one with the highest Tm corresponds with the unfolding of a nine-basepair long central hairpin. Dimethylation of the adenosines in the loop of this hairpin lowers the melting temperature (Tm) by approximately 20°C at 0.2 M NaCl and by about 5°C at 0.015 M NaCl. It is suggested that m2Am2A is more antagonistic to loop formation than ApA and that the function of the methyl groups is to help to destabilize the 3'-terminal hairpin in 16S rRNA in order to facilitate intermolecular interactions.

INTRODUCTION

Ribosomal RNA's are highly organized molecules containing multiple hairpins and long distance interactions (1-3). These RNA's are assumed to participate, in cooperation with ribosomal proteins, actively in the individual steps of protein biosynthesis. One of the parts of the 16S ribosomal RNA for which specific functions have been proposed is the 3'-end (4, 5). There is evidence that nucleotides near the 3'-terminus participate in initiation of protein synthesis by basepairing with initiation sites on messenger RNA (5).

A remarkable feature of the RNA of small ribosomal subunits, both in prokaryotes and eukaryotes, is the occurrence of two adjacent N6-dimethyladenines (m2Am2A) at 20 to 25 nucleotides from the 3'-terminus. Although the strong conservation of this modification points to an important role, a mutant of Escherichia coli, resistant towards the antibiotic kasugamycin, lacks the enzyme that specifically methylates these two adenosines in wild-type bacteria (6). This mutant grows with the same rate as wild-type E. coli and
indeed, elaborate in vitro studies have shown that only very small, although
distinct differences in activity between the two types of ribosomes can be
detected (7,8). These differences are the slightly enhanced amount of the
initiation factor IF-3 that is required for maximal binding of formyl-methio-
nyl-tRNA to mRNA programmed mutant 30S ribosomes (7) and a decreased affinity
of these subunits to associate with 50S particles (8).

In order to fully understand the mechanism by which the methylgroups act,
precise knowledge of their effects on the local conformation of the ribosome
would be required. As a first approximation it would be useful to study the
effect of the methylgroups on the structure of an isolated part of the ribo-
sonal RNA, inОџ u the 49 nucleotide fragment that is cleaved off from 16S
ribosomal RNA by treatment of the ribosomes with colicin E3 or cloacin DF13
(9). Studies with physical techniques of the fragment from wild-type ribo-
sones (10,11) have confirmed the existence of a central hairpin structure
with the dimethyl-A's in the loop, as first proposed on the basis of nucleo-
tide sequence and as illustrated in Fig. 1A. It has also been claimed that
further basepairing interactions exist as shown in Fig. 1B or C (11,12). We
have shown recently (13) that the conformational stability of the 49 nucleo-
tide fragment is influenced by the methylgroups. This was deduced from the
effect of the methylgroups on the electrophoretic mobility in gels in the
presence of urea. In this paper it is shown that dimethylation of the A's
results in a destabilization of the central hairpin structure.

MATERIALS AND METHODS

Materials. E. coli strain PR7 and its kasugamycin resistant derivative
TPR201 were kindly provided by Dr. J.E. Davies (Univ. of Wisconsin, Madison,
U.S.A.). Dr. F.K. De Graaf generously provided cloacin.

Isolation of the cloacin fragments. The isolation of the cloacin frag-
ments was according to the procedure described by Baan et al. (9). Each time
an aliquot of the final preparation was taken, labeled with (y-32)ATP and
sequenced to establish its intactness and purity. In order to remove Mg2+
ions, solutions of the purified fragments were passed through G25, equili-
brated with 2 M NaCl; hereafter the solutions were desalted on G25.

Melting experiments. During the melting experiments the absorbance at
\( \lambda = 260 \text{ nm} \) of the RNA samples were measured on a Cary 118 spectrophotometer
using a constant temperature cell (Hellma). The temperature was controlled by
circulating water from a Colora cryothermostat (Mettler, WK 5) first through
Figure 1. Alternative structures of the 3'-terminus of 16S rRNA. The fragment represented results from treatment of 70S ribosomes with colicin E₃ or cloacin DF₁₃.

a thermostattable cell holder and then through the cell. The cryothermostat was modified in order to be able to vary the heating rate from 0.02 to 1.5°C per minute. The temperature was measured with a thermolinear temperature probe (YSI, 702). It was placed in the cell and served as a feedback resistor for an operational amplifier. The output of this amplifier varies from 0 to 1 volt when temperature changes from 0 to 100°C. Measurement of the absorbance
and temperature was carried out automatically using a dual programmable electronic switch connecting either the Cary 118 output or the output of the thermistor amplifier with a digital voltmeter (Data precision). The digital voltmeter was interfaced to a table computer (Diehl, alphatronic, DS 300). A schematic representation of the repeating cycle measuring the absorbance and temperature is shown in Fig. 2.

RESULTS

1. Melting transitions of the 49 nucleotide fragment from the mutant strain

Little is known of the influence of base-modification on the thermal stability of RNA secondary structure. Here we have a relatively small RNA of 49 nucleotides with 6 (wild-type) and 2 (mutant) additional methyl groups, respectively. The data obtained with the mutant RNA fragment are discussed first.

Fig. 3A shows optical melting curves of mutant 49 nucleotide fragments at three different NaCl concentrations. The meltings are almost perfectly reversible since on slow cooling the curves are virtually super-imposable and the starting optical densities are regained within an error of one percent (data not shown). An aliquot of each sample was labeled at the 5'-end with $^{32}P$ before and after the melting experiments and the intactness of the RNA's was analyzed by electrophoresis in the presence of urea. In no case could more than a few percentages of contamination or degradation be observed (results not shown).

```
| start | end   |
|------|------|
| of   | of   |
| cycle| cycle|
| temp | temp |
| ↑    | ↑    |
| ↓    | ↓    |
| 0    | 36   |
| 2    | 38   |
| 4    | 40   |
| time (s) | 32 measurements of A |
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Figure 2. At $t = 2$ s the temperature of the solution in the cell is measured followed by 32 measurements of A at intervals of 1 s. At $t = 37$ s these 32 measurements are averaged, at $t = 38$ s the temperature is remeasured and at $t = 39$ s this value and the value obtained at $t = 2$ s are averaged. These results served as one set of values for A and T. The experimental data were fed to a computer (IBM 370/10). Subsequently a third degree polynomial was fitted to each 9 succeeding experimental points using the subroutine RELOTH (IMS Library) $\Delta A/\Delta T$ vs T values were calculated by the computer as the first derivative of the polynomial.
Figure 3. (A) Corrected hyperchromic melting profile at 260 nm of the 49 nucleotide fragment derived from the 3′-end of 16S rRNA of ribosomes from the strain TPR201 at different NaCl concentrations.
(B) Differentials of the hyperchromism profile at different NaCl concentrations. (---) 0.015 M NaCl; (- - -) 0.1 M NaCl; (--- -) 0.2 M NaCl.

The total hyperchromicity at 260 nm observed when the samples are heated from 4°C to 84°C is independent of the salt concentration and amounts to 27 ± 0.4%. When we assume that at 84°C the RNA's are completely unfolded at these salt concentrations, the same number of basepairs are present at 4°C at each Na⁺ concentration. From data in the literature on the hypochromic effects of base-stacking and basepairing (14) the total hyperchromicity for the unfolding of the three secondary structures depicted in Fig. 1 into the random coil can be calculated. Structure A would yield a hyperchromic effect of 16.8%; structure B of 24.7% and structure C of 26.9% at 260 nm. Structures B and C therefore would give values that are close to the experimental data.

The melting curves shown in Fig. 3A were differentiated using a computer program (compare Materials and Methods) and the plots of $\Delta A_{260}/A_T^1$ versus T are shown in Fig. 3B. Exactly the same data were obtained several times with different, independent isolates of the fragment (compare also Materials and Methods). Three distinct transitions are visible at 0.015 M NaCl; they are
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designated I, II and III at T_m's of 30°C, 39°C and 57°C, respectively in Fig. 3B. The transitions I and II together comprise approximately 50% of the hyperchromicity and transition III about the other half. The differential melting curve at 0.100 M NaCl is quite similar to the one at 0.015 M NaCl, except that all transitions have shifted to higher T_m's (I: 47°C; II: 56°C; and III: 71°C). The whole pattern is shifted to still higher temperatures at 0.200 M NaCl and the transitions I and II almost converge, prohibiting an exact determination of their T_m's (I: ~ 55°C; II: ~ 60°C and III: 75°C).

In the low salt range (0.015-0.100 M NaCl) all three transitions show a very similar, linear salt dependence of T_m: \( \frac{dT_m}{d\log(\text{Na}^+)} = 17.5-19.5°C \).

The presence of the basepaired regions in Fig. 1A in the wild-type molecule has been demonstrated by NMR (10) and by T-jump relaxation kinetics (11). In the latter case, a T_m of 73.5°C was reported at 0.1 M Na⁺ for the main melting transition. No doubt the main peak in the profiles of Fig. 3B corresponds to the melting of the 9 basepair long helix present in all three secondary structure models of Fig. 1. Although it is tempting to assign the other two melting transitions to the structure as in Fig. 1C, this should await further data obtained by other techniques.

2. Comparisons of the melting transition of wild-type (methylated) and mutant (unmethylated) fragments

Fig. 4 shows the melting curves and their differentiated forms (\( \Delta A/\Delta T \) versus T) of wild-type and mutant fragment at 0.015 M NaCl (Fig. 4A, C) and at 0.2 M NaCl (Fig. 4B, D). The total hyperchromicity accompanying the melting of the wild-type fragment is roughly the same as with the mutant molecule and therefore points to the presence of the structures of Fig. 1B or C. At 0.015 M NaCl three melting transitions are observable with T_m's of 30°C, 41.5°C and 52°C. Hence two of the transitions occur with a T_m that is different from the ones observed in the mutant fragment. The observation which we would like to emphasize, however, is the lowering of the main melting transitions from 57°C to 52°C at 0.015 M NaCl and from 75°C to 73°C at 0.2 M NaCl upon methylation of the adenines. The \( \frac{dT_m}{d\log(\text{Na}^+)} \) (compare Fig. 5) for the main transition in the wild-type fragment is virtually the same as that for the mutant in the range 0.015 to 0.2 M NaCl.

As in our previous studies (8,13) all the effects measured were unambiguously ascribed to the methyl groups by doing control experiments with fragments obtained from in vitro methylated mutant ribosomes.

The differentiated melting curve can be used to calculate the enthalpy
Figure 4. Corrected hyperchromic melting profiles (A, B) and differentials of the hyperchromism profiles (C, D) at 260 nm of the 49 nucleotide fragment derived from the 3'-end of 16S rRNA of wild-type (-----) and mutant (------) ribosomes at 0.015 M NaCl (A, C) and at 0.2 M NaCl (B, D).

of the transition with the highest $T_m$ (transition III) using the equation (15): $\Delta H = \frac{-3.2}{\frac{1}{T_h} - \frac{1}{T_k}}$.

In this equation $T_h$ is the temperature at the maximum which is equal to the melting temperature $T_m$ and $T_k$ the temperature at the upper half height of the derivative of the melting curve. Subsequently the entropy of the melting transition can be calculated since at the melting temperature $T_m = \Delta H/\Delta S$.

Figure 5. Dependence of $T_m$ of the different transitions (compare Fig. 3) of the 49 nucleotide fragment derived from the mutant (●●●) and of transition III of the 49 nucleotide fragment derived from the wild-type (O---O) upon the ionic strength.
Results of the calculations are listed in Table 1.

DISCUSSION

The results described here show that the 49 nucleotide fragment derived from the 3'-end of the 16S ribosomal RNA (colicin or cloacin fragment) contains except for a major helical region (10,11) two additional structured regions. Although structure C of Fig. 1 is qualitatively consistent with these observations, definite assignment of the two low temperature melting transitions must await further experiments with other techniques. Differential melting curves for the wild-type fragment were also measured by Yuan et al. (11) using T-jump relaxation kinetics. In addition to the transition coming from the major helical region these investigators found another transition around 20°C which could be consistent with the existence of either structure B or C in Fig. 1. The melting temperature of this transition is, however, lower than the ones we found. The melting temperatures of the central helix are somewhat higher in the T-jump experiments than in our equilibrium measurements while the melting enthalpy is found slightly higher in the kinetic experiments. In view of the different methods used the agreement is reasonable, however. The salt dependence of the major transition \(dT_m/d\log(\text{Na}^+) = 18-20^\circ\text{C}\) is in the range of the values determined for hairpin structures of transfer RNA (16).

Despite the accuracy of our melting experiments the differences in the thermodynamic parameters characterizing the melting of the wild-type and the mutant fragment cannot be used to draw definitive conclusions with regard to the molecular basis for the difference in thermal stability of the two species. However, our experiments definitely show that the central helix is less stable in the wild-type than in the mutant fragment. Since at the melting temperature the free enthalpy of hairpin formation is zero it can easily be shown that for the formation of the central hairpin

Table 1. Thermodynamic parameters for the formation of the major hairpin in the cloacin fragments of 16S rRNA at 0.2 M NaCl. The values are the average of four independent measurements.

|          | Mutant | Wild-type |
|----------|--------|-----------|
| \(T_m\) (°C) | 75.0   | 72.8      |
| \(\Delta H^\circ\) (kcal/mole) | -61.2  | -53.4     |
| \(\Delta S^\circ\) (kcal/mole K) | -0.176 | -0.154    |
\[
\Delta S^w_{(\text{loop})} - \Delta S^m_{(\text{loop})} = \frac{\Delta T_m}{T_w^\circ} \cdot \Delta S^m
\]

where \(\Delta S^w_{(\text{loop})}\) is the entropy of loop formation of the wild type and \(\Delta S^m_{(\text{loop})}\) the entropy of loop formation of the mutant central hairpin; \(\Delta T_m\) is the difference in the melting temperature of the mutant and the wild-type central hairpin; \(T_w^\circ\) the melting temperature of the wild-type hairpin (in K) and \(\Delta S^m\) is the entropy of central hairpin formation in the mutant. In the derivation of this equation it was assumed that the enthalpy and entropy of helix formation of both species are equal. Moreover it was assumed that the difference between the enthalpy of loop formation for the two species is zero. This assumption is somewhat less restrictive than the usual assumption that the enthalpy of loop formation is zero. On the basis of this we find: \(\Delta S^w_{(\text{loop})} - \Delta S^m_{(\text{loop})} = -1.1 \text{ cal/mole K} \).

It is interesting to compare our results with the properties of ApA and \(\text{m}_2\text{Apm}_2\text{A}\) (17,18). It was found that dimethylation results in an increase of the stacked form of the dinucleotide from about 50\% for ApA to about 80\% for \(\text{m}_2\text{Apm}_2\text{A}\). This is mainly due to a slightly less unfavorable entropy change in stacking the bases of \(\text{m}_2\text{Apm}_2\text{A}\) than of ApA which amounted to -1.7 and -3.4 cal/mole K, respectively (17,18). These values are close to the value obtained for the difference in entropy of loop formation of the two 49 nucleotide fragments. Apparently also in the loop the \(\text{m}_2\text{Apm}_2\text{A}\) part has a higher tendency to stack than the ApA part and this increased stacking then leads to a somewhat less stable hairpin in the wild-type fragment.

What could this destabilization possibly mean for the function of the ribosome? There is rather strong evidence that the 3'-end of 16S RNA is involved in initiation (5) and in subunit interaction (8,19). Both events are very slightly disturbed \textit{in vitro} when the ribosome is lacking the methylgroups (7,8). In both cases it has been suggested that the involvement of the 3'-end of the RNA is through intermolecular RNA-RNA interactions. These interactions could benefit from the transient opening and closing of the secondary structure at the 3'-end of the RNA. In the fine-tuning of this mechanism the methylgroups could play a role, either directly by destabilization of the intramolecular secondary structure, or indirectly by functioning as a recognition mark for one or more proteins or by a combination of these possibilities. Especially the functions of the proteins S21, IF-3 and IF-1 are somehow connected with the role of the methylgroups and with initiation of protein synthesis and subunit interaction (7,8).

Recently we showed that the methylgroups influence the electrophoretic
mobilities of nucleotide fragments derived from the 3'-end rather strongly on polyacrylamide gels in the presence of urea (13). Urea solutions act as hydrophobic solvents in the denaturation of proteins (20) and it is reasonable to assume that they do the same with nucleic acids. Since in hydrophobic solvents bases lose their tendency to stack but keep their pairing properties through hydrogen bonding (21), the observations in that study are best explained by the fact that N\(^6\)-dimethyladenine cannot hydrogen bond to uracil.

REFERENCES

1. Woese, C.R., Magrum, L.J., Gupta, R., Siegel, R.B., Stahl, D.A., Kop, J., Crawford, N., Brosius, J., Gutell, R., Hogan, J.J. and Noller, H.F. (1980) Nucleic Acids Res. 8, 2275-2293.
2. Glotz, G. and Brimacombe, R. (1980) Nucleic Acids Res. 8, 2377-2395.
3. Stiegler, P., Carbon, P., Zuker, M., Ebel, J.P. and Ehresmann, C. (1981) Nucleic Acids Res. 9, 2153-2172.
4. Shine, J. and Dalgarno, L. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 1342-1346.
5. Steitz, J.A. and Jakes, K. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 4734-4738.
6. Helser, T.L., Davies, J.E. and Dahlberg, J.E. (1971) Nature New Biol. 233, 12-14.
7. Poldermans, B., Van Buul, C.P.J.J. and Van Knippenberg, P.H. (1979) J. Biol. Chem. 254, 9090-9093.
8. Poldermans, B., Bakker, H. and Van Knippenberg, P.H. (1980) Nucleic Acids Res. 8, 143-151.
9. Baan, R.A., Van Charldorp, R., Van Leerdam, E., Van Knippenberg, P.H., Bosch, L., De Rooij, J.F.M. and Van Boom, J.H. (1976) FEBS Lett. 71, 351-355.
10. Baan, R.A., Hilbers, C.W., Van Charldorp, R., Van Leerdam, E., Van Knippenberg, P.H. and Bosch, L. (1981) Nucleic Acids Res. 9, 1028-1031.
11. Yuan, R.C., Steitz, J.A., Moore, P.B. and Crothers, D.M. (1979) Nucleic Acids Res. 7, 2399-2418.
12. Dahlberg, A.E. and Dahlberg, J.E. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 2940-2944.
13. Van Charldorp, R., Heus, H.A. and Van Knippenberg, P.H. (1981) Nucleic Acids Res. 9, 267-275.
14. Richards, E.G. in: "Handbook of Biochemistry and Molecular Biology", 3rd edition (G.D. Fasman, ed.), Nucleic Acids, Vol. I, pp. 596-603, CRC-Press, Cleveland, Ohio.
15. Gralla, J. and Crothers, D.M. (1973) J. Mol. Biol. 78, 301-319.
16. Cole, P.E., Yang, S.K. and Crothers, D.M. (1972) Biochemistry 11, 4358-4368.
17. Olsthoorn, C.S.M., Haasnoot, C.A.G. and Altona, C. (1980) Eur. J. Biochem. 106, 85-95.
18. Tazawa, I., Kaine, T. and Inoue, Y. (1980) Eur. J. Biochem. 109, 33-38.
19. Van Duin, J., Kurland, C.G., Dondon, J., Grunberg-Manago, M., Branlant, C. and Ebel, J.P. (1976) FEBS Lett. 62, 111-114.
20. Creighton, T.E. (1979) J. Mol. Biol. 129, 235-264.
21. Ts'O, P.O.P., in: "Basic Principles in Nucleic Acid Chemistry", Vol. I, Chapter 6, Academic Press Inc., New York.