The Effect of Hypermethylation on the Functional Properties of Transfer Ribonucleic Acid

RIBOSOME-BINDING AND POLYPEPTIDE SYNTHESIS

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1. Phenylalanyl-tRNA formed after chemical hypermethylation of *Escherichia coli* B tRNA was able to bind to ribosomes with the same efficiency as normal phenylalanyl-tRNA. 2. Under incubation conditions used in the ribosome-binding assay, hypermethylation of tRNA did not measurably decrease the stability of either internucleotide phosphodiester bonds or the covalent bond between amino acid and tRNA in phenylalanyl-tRNA. 3. The ability of hypermethylated tRNA to take part in polyphenylalanine synthesis was inhibited progressively as the degree of hypermethylation increased. 4. Hypermethylation of tRNA affected polyphenylalanine synthesis at the stage of amino acid recognition and at a further point in the synthesis but not at the level of codon-anticodon recognition. 5. The formation of polylysine was more seriously affected by hypermethylation of tRNA than would be accounted for by inhibition of amino acid acceptance alone. 6. Polyproline formation was completely inhibited by the presence of 7 mol % excess of methyl groups in tRNA. 7. The possibility of a link between amino acid acceptance and ribosome-binding was suggested for phenylalanyl-tRNA, but not for lysyl- or prolyl-tRNA.

There have been several studies on the chemical modification of bases in tRNA, and the resulting change in the biological activity of these tRNA species has been correlated with alterations in configuration or base sequence at specific sites in the molecule (Miura, 1967). In this context, modification of tRNA by chemical or enzymic hypermethylation is of particular interest since it has been shown that, in tumour tissue, tRNA has an abnormal methylated base content (Borek, 1969).

In a previous paper (Pillinger, Hay & Borek, 1969) we have described the effect of hypermethylation of tRNA with dimethylsulphate, under conditions in which 7-methylguanine is formed predominantly, on its ability to form aminoacyl-tRNA *in vitro*. This study has now been extended and the performance of such hypermethylated tRNA in a ribosome-binding assay (Nirenberg & Leder, 1964) and a polypeptide-synthesizing system is discussed.

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A preliminary account of some of this work has appeared (Hay, 1969).

MATERIALS AND METHODS

Polynucleotides, oligonucleotides and amino acids. Poly A, poly C, poly U and poly G and the trinucleoside diphosphates, UpUpU and CpCpC were obtained from Miles Chemical Co., Elkhart, Ind., U.S.A.

Radioactive amino acids were obtained from the New England Nuclear Corp., Boston, Mass., U.S.A. with the following specific radioactivities ($\mu\text{Ci}/\mu\text{mol}$): [^{14}C]lysine, 247; [^{14}C]phenylalanine, 297; [^{14}C]proline, 190; [^{14}C]glycine, 100.

tRNA and hypermethylated tRNA. *Escherichia coli* B tRNA was purchased from General Biochemicals Inc., Chagrin Falls, Ohio, U.S.A., and chemically methylated and purified as outlined by Pillinger *et al.* (1969). Purified phenylalanine tRNA from *E. coli* B was a gift from Dr G. D. Novelli, Oak Ridge, Tenn., U.S.A.; 88% of its acceptance ability was specific for phenylalanine.

Preparation of ribosomes and supernatant fractions Ribosomes were prepared from either *E. coli* B or *E. coli* Q13 cells in exponential phase, and washed three times with 2M-KCl at 0-4°C as described by Söll *et al.* (1966).

Supernatant fractions of these cells were freed of tRNA by passage through a DEAE-cellulose column and

concentrated by using polyethylene glycol (Söll & RajBhandary, 1967). Neither ribosomes nor supernatant fractions were frozen before use, but, on occasion, were prepared, with a small loss in activity, from frozen cells.

Formation of aminoacyl-tRNA. Normal or hypermethylated *E. coli* B phenylalanine tRNA was incubated with a tRNA-free aminoacyl-tRNA synthetase preparation from the same cells under conditions described by Pillinger *et al.* (1969). The [^{14}C]phenylalanyl-tRNA so formed was purified by phenol extraction at pH 4.5, ethanol precipitation in the presence of 2% (w/v) potassium acetate and dialysis against 0.02 M-sodium acetate buffer, pH 4.5 and 2 mM-MgCl₂ for 6 h at 0°C.

Ribosome-binding assay. The ability of phenylalanyl-tRNA to bind to washed ribosomes in the presence of trinucleoside diphosphates was carried out as described by Nirenberg & Leder (1964).

Amino acid incorporation in vitro. The reaction mixture was as follows: 60 mM-tris-HCl buffer, pH 7.5, 10 mM-magnesium acetate, 0.5 mM-MnCl₂, 12 mM- β -mercaptoethanol, 50 mM-NH₄Cl, 0.25 mM-GTP, 2 mM-ATP, 5 mM-sodium phosphoenolpyruvate, 20 μg of phosphoenolpyruvate kinase/ml, 2 μCi of ^{14}C -labelled amino acid/ml, 17 E_{260} units of washed ribosomes, template polymer (0.1 mM) and 0.5–1.0 mg of supernatant protein. tRNA was added to the incubation mixtures in the amounts given in the figure legends. Incubation was carried out at 37°C.

The polypeptide synthesized was assayed by trichloroacetic acid-insolubility (0.25% tungstic acid was added for polylysine) by using the filter-disc method of Morgan, Wells & Khorana (1967). Exploratory experiments were carried out with this technique to ensure that all the aminoacyl-tRNA that had been formed was destroyed before radioactivity was assayed.

RESULTS

Stability of hypermethylated and normal aminoacyl-tRNA. We had previously shown that the ability of a purified *E. coli* B phenylalanine tRNA preparation to form phenylalanyl-tRNA *in vitro* was inhibited by 50% when the molecules contained 7 mol % excess of methyl groups. (Pillinger *et al.* 1969). Consequently, it was important to determine to what extent these tRNA molecules, which were still able to accept amino acid after methylation, retained an ability to participate in codon-anticodon recognition. The presence of additional methyl groups in the structure of aminoacyl-tRNA may well have profound effects on the chemical stability of the molecule. It seemed worth while, therefore, to examine this stability under the conditions used in the ribosome-binding assay before such assays were attempted.

If instability were introduced into the tRNA molecule, it could result in cleavage of phosphodiester bonds to give rise to fragments ranging in size from mononucleotides to large oligonucleotides. Also, in aminoacyl-tRNA, the covalent bond linking the amino acid to the adenosine of the tRNA

could be affected by hypermethylation. Any of these possibilities could affect the ribosome-binding ability of tRNA.

E. coli B tRNA hypermethylated to various extents up to 13 mol % excess of methyl groups (Pillinger *et al.* 1969) and normal *E. coli* B tRNA were incubated under ribosome-binding assay conditions for up to 60 min. Small volumes of each sample were applied to DEAE-cellulose paper before being subjected to ascending chromatography in ammonium hydrogen carbonate solutions of concentrations up to 0.8 M.

In this assay, fragments of tRNA move more rapidly with the solvent than do the intact molecules and can be readily detected (J. Hay & D. J. Pillinger, unpublished work). Results of these experiments indicated that the hypermethylated tRNA molecules behaved identically with normal tRNA.

Next, the release of acid-soluble fragments from 3'-OH terminus of [^{14}C]phenylalanyl-tRNA was followed under ribosome-binding assay conditions with both normal and hypermethylated tRNA. The result is given in Fig. 1 and showed that both preparations behaved similarly.

Fig. 1. Stability of normal and hypermethylated phenylalanyl-tRNA. Normal and hypermethylated (7 mol % excess of methyl groups) [^{14}C]phenylalanyl-tRNA preparations were incubated at 37°C under the binding-assay conditions of Nirenberg & Leder (1964) for the times shown. Samples were transferred into 3 ml of 5% (w/v) trichloroacetic acid containing 100 μg of heat-denatured DNA, at 4°C. After 10 min the precipitated material was gathered on a Millipore filter which was washed four times with 5 ml of 5% (w/v) trichloroacetic acid, dried at 37°C and added to 10 ml of 0.4% 2,5-diphenyloxazole in toluene for scintillation-counting. ●, Phenylalanyl-tRNA; ○, hypermethylated phenylalanyl-tRNA.

Table 1. Binding of normal and hypermethylated phenylalanyl-tRNA to ribosomes

The incubation conditions were as described by Nirenberg & Leder (1964). In Expts. I and III phenylalanine tRNA was added to the normal [¹⁴C]phenylalanyl-tRNA preparation such that its specific radioactivity was equal to that of the hypermethylated phenylalanyl-tRNA preparation. In Expt. II the specific radioactivity of the normal phenylalanyl-tRNA preparation was 2.5 times that of the hypermethylated sample.

tRNA	Triplet	Aminoacyl-tRNA bound (pmol)		
		Expt. 1	Expt. 2	Expt. 3
Phenylalanyl-	UpUpU	0.30	1.48	0.31
Phenylalanyl-	CpCpC	0.28	0.79	0.19
Methylated phenylalanyl-	UpUpU	0.61	1.47	0.85
Methylated phenylalanyl-	CpCpC	0.45	0.82	0.55
Phenylalanyl-	—	0.24	0.75	0.21
Methylated phenylalanyl-	—	0.43	0.83	0.65

These results indicated that hypermethylation of tRNA gave rise to no observable difference in its chemical stability under the conditions employed.

Binding of hypermethylated tRNA to ribosomes. Phenylalanyl-tRNA, formed *in vitro* with ¹⁴C-labelled phenylalanine and normal and hypermethylated tRNA, was tested in the ribosome-binding assay. As noted by Pillinger *et al.* (1969), hypermethylated phenylalanyl-tRNA is less able than normal tRNA to accept amino acid. Thus, the ratio of the specific radioactivities of the aminoacyl-tRNA species (normal hypermethylated) was of the order of 2:1. For this reason binding experiments were carried out with these aminoacyl-tRNA preparations and also with tRNA preparations whose specific radioactivities had been adjusted by addition of unlabelled phenylalanine tRNA to the 'normal' preparation. The same proportion of amino acid residues was then present in both samples. The results are given in Table 1.

Under conditions in which the specific radioactivities of both phenylalanyl-tRNA species were equal, the hypermethylated material bound to ribosomes twice as well as the normal tRNA. However, when aminoacyl-tRNA species were used with the different specific radioactivities that arose directly from the effect of methylation, the incorporation of [¹⁴C]phenylalanyl-tRNA into stable complexes with ribosomes was identical in both cases. The results therefore suggest that there is little or no competition between hypermethylated aminoacyl-tRNA and hypermethylated non-acylated tRNA in this ribosome-binding system. The effect of hypermethylation on ribosome-binding was not, in this case, similar to its effect on amino acid acceptance.

Synthesis of poly-L-phenylalanine. The purified phenylalanine tRNA that had been assayed in the aminoacylation system and the ribosome-binding test was now used in a study of its ability to take part in polyphenylalanine synthesis *in vitro*. The

Fig. 2. Effect of hypermethylation of phenylalanine tRNA on its function in polyphenylalanine formation. Unfractionated *E. coli* B tRNA, both normal and hypermethylated (7mol % excess of methyl groups) was incubated in the poly U-directed polyphenylalanine-synthesizing system described in the Materials and Methods section. The final concentration of tRNA was 1.2mg/ml. ●, Normal tRNA; ■, hypermethylated tRNA; ▲, no added tRNA; ○, no added template.

system used was entirely dependent on added tRNA and added template. *E. coli* B tRNA, both normal and hypermethylated (7mol % excess of methyl groups), was incubated in the polypeptide-synthesizing system with poly U for the times shown in Fig. 2. It is clear that the presence of excess of methyl groups in the tRNA has a marked effect on its function, and its ability to participate in

Fig. 3. Effect of increasing hypermethylation of phenylalanine tRNA on its ability to function in polyphenylalanine synthesis. Normal unfractionated *E. coli* B tRNA and three hypermethylated *E. coli* B tRNA preparations (7, 10 and 13 mol % excess of methyl groups) were added at a final concentration of 1.2mg/ml to the poly U-directed polyphenylalanine-synthesizing system described in the Materials and Methods section. ●, Normal tRNA; hypermethylated tRNA with 7 (■), 10 (○) and 13 (Δ) mol % excess of methyl groups; ▲, no added tRNA.

polypeptide synthesis was at least four times less than its normally methylated counterpart.

That the relative inability of hypermethylated tRNA to form polyphenylalanine *in vitro* was a function of the extent of hypermethylation is shown in Fig. 3, in which three hypermethylated tRNA preparations (7, 10 and 13 mol % excess of methyl groups) were examined. There was a marked fall in activity of the tRNA as methylation increased and this effect was similar, in direction if not in magnitude, to that observed for amino acid acceptance (Pillinger *et al.* 1969). At 13 mol % excess of methyl groups essentially all activity had been lost. To obtain an accurate value for the inactivation of phenylalanine tRNA several concentrations of normal and hypermethylated (7 mol % excess of methyl groups) *E. coli* B tRNA preparations were used in polyphenylalanine formation. The results (Fig. 4) show that the true degree of inhibition of polyphenylalanine synthesis after hypermethylation is greater than 80%. Increasing the amount of hypermethylated tRNA produced a linear increase in the amount of polypeptide formed, up to and beyond that at which normally methylated tRNA had become saturating.

Parallel experiments with purified phenylalanine tRNA and with mixed aminoacyl-tRNA

Fig. 4. Polyphenylalanine formation as a function of the concentration of normal and of hypermethylated tRNA. The indicated amounts of normal and hypermethylated (7 mol % excess of methyl groups) *E. coli* B tRNA were added to the poly U-directed polyphenylalanine-synthesizing system described in the Materials and Methods section, during a 10 min incubation at 37°C. ●, Normal tRNA; ■, hypermethylated tRNA.

Fig. 5. Effect of hypermethylation of lysine tRNA on its function in polylysine formation. Unfractionated *E. coli* B tRNA preparations, both normal and hypermethylated (7 mol % excess of methyl groups) were incubated in the poly A-directed polylysine-synthesizing system described in the Materials and Methods section. The final concentration of tRNA was 1.2mg/ml. ●, Normal tRNA; ■, hypermethylated tRNA; ○, no added template; ▲, no added tRNA.

species indicated that both preparations behaved identically in the above system.

Formation of poly-L-lysine. Fig. 5 shows the effect of hypermethylation of tRNA (7 mol % excess of methyl groups) on its ability to form polylysine *in vitro*. The system was identical with

Fig. 6. Polylysine formation as a function of the concentration of normal and of hypermethylated tRNA. The indicated amounts of normal and hypermethylated (7mol % excess of methyl groups) *E. coli* B tRNA were added to the poly A-directed polylysine-synthesizing system, described in the Materials and Methods section, during a 10 min incubation at 37°C. ●, Normal tRNA; ■, hypermethylated tRNA.

Fig. 7. Effect of hypermethylation of proline tRNA on its function in polypeptide formation. Unfractionated *E. coli* B tRNA preparations, both normal and hypermethylated (7mol % excess of methyl groups) were incubated in the poly C-directed polypeptide-synthesizing system described in the Materials and Methods section. The final concentration of tRNA was 1.2mg/ml. ●, Normal tRNA; ■, hypermethylated tRNA; △, no added tRNA; ○, no added template.

that used for polyphenylalanine synthesis, except that lysine and poly A replaced phenylalanine and poly U. Again, there was a marked decrease in the formation of polylysine after methylation of the tRNA. An absolute value for this inhibition was obtained from tRNA concentration curves (Fig. 6); this was of the order of 60%, somewhat less than for the polyphenylalanine system.

Formation of poly-L-proline. Polyproline was prepared by using poly C *in vitro* in the presence of normal and hypermethylated tRNA as described for the two polypeptide syntheses above. Again, two curves were obtained. In Fig. 7, the effect of incubation time on polyproline formation indicated that the presence of 7mol % excess of methyl groups was sufficient to destroy completely the

Fig. 8. Polypeptide formation as a function of the concentration of normal and of hypermethylated tRNA. The indicated amounts of normal and hypermethylated (7mol % excess of methyl groups) *E. coli* B tRNA were added to the poly C-directed polypeptide-synthesizing system, described in the Materials and Methods section, during a 10 min incubation at 37°C. ●, Normal tRNA; ■, hypermethylated tRNA.

Table 2. Comparison of the effect of hypermethylation of tRNA on its ability to function in amino acid-acceptance and in polypeptide synthesis

Amino acid	% inhibition of function	
	Formation of aminoacyl-tRNA*	Formation of polypeptide†
Lysine	22	60
Phenylalanine	50	82
Proline	50	100

* Derived from Pillinger *et al.* (1969).

† Derived from Figs. 4, 6 and 8.

ability of the tRNA to take part in polypeptide synthesis. The second curve, which explored the effect of increasing tRNA concentration on the system (Fig. 8), confirmed the finding in Fig. 7. Even with a concentration of hypermethylated tRNA 50% greater than that employed as a routine in this assay there was no indication of polypeptide synthesis. The extent of inhibition of polypeptide synthesis was greater than that of either polyphenylalanine or polylysine.

Comparison of aminoacylation and polypeptide synthesis. Table 2 lists the extent of inhibition of aminoacylation and of polypeptide-synthesizing ability caused by methylation (7mol % excess of methyl groups) of tRNA. In each case it is apparent that polypeptide synthesis was much more sensitive to hypermethylation than was amino acid acceptance.

Aminoacylation of lysine tRNA was least affected by hypermethylation. It may be significant that polylysine formation was also the least sensitive

(of the three syntheses studied) to the presence of hypermethylated tRNA.

Quantitative differences in polypeptide-synthesizing ability were always observed among extracts of *E. coli* B cells despite efforts to harvest cells at the same phase of exponential growth (cf. Figs. 2 and 4). However, experiments carried out with cell extracts with different activities gave quite consistent results. The synthesis of proline was much less efficient than that of polyphenylalanine or polylysine and this appears chiefly to be due to a low affinity of poly C for ribosomes (Wahba *et al.* 1963).

DISCUSSION

Binding of hypermethylated tRNA to ribosomes.

The hypermethylated phenylalanyl-tRNA preparation used in the binding assay contained approx. 50% of the concentration of aminoacylated molecules of the normally methylated phenylalanyl-tRNA. That only 50% of the hypermethylated phenylalanine tRNA preparation can be aminoacylated is presumably due to the presence of methyl groups at critical points in the tRNA molecules (Pillinger *et al.* 1969). Despite this, however, it appears that hypermethylated phenylalanyl-tRNA is able to bind to washed *E. coli* ribosomes to the same extent as normally methylated phenylalanyl-tRNA under conditions in which the tRNA is not saturating.

The ribosome-binding experiments show that the hypermethylated tRNA molecules that can be aminoacylated are fully able to form stable complexes with ribosomes in the presence of UpUpU. Since the presence in the hypermethylated tRNA preparation of hypermethylated but non-acylated molecules does not diminish the binding of molecules that have been aminoacylated, it seems likely that there can be no competition for the binding site(s) from the hypermethylated non-acylated tRNA. Thus, apparently, phenylalanine tRNA molecules that have been inactivated in their aminoacylation function have also been inactivated in ribosome-binding ability. The converse of this, of course, does not necessarily follow.

One explanation for the failure of a hypermethylated tRNA molecule to bind to a ribosome or to compete with other molecules for the binding site would be that extra methyl groups have been inserted in the anticodon triplet or at some other point in the anticodon region. Gefer & Russell (1969) and Thiede & Zachau (1968) have shown that in *E. coli* tyrosine suppressor tRNA and in yeast phenylalanine tRNA respectively removal (or partial removal) of modification to a base in this region of the molecule has profound effects on ribosome-binding ability.

These results, then are not incompatible with the existence of a link at the molecular level between the amino acid-acceptance ability of tRNA and its ability to bind to ribosomes. This latter function appears primarily to involve the anticodon region of the molecule, whereas there is still much speculation as to the nature of the site(s) responsible for amino acid-acceptance, although the 3'-terminal region is known to be involved (Hecht, Zamecnik, Stephenson & Scott, 1958). Schulman & Chambers (1968) have presented convincing evidence that in yeast alanyl-tRNA the specificity for aminoacylation resides in the helical region containing the 3'- and 5'-terminal sequences.

Hypermethylation may exert its effect on both amino acid-acceptance and on ribosome-binding by a general disruption of the secondary or tertiary structure of the molecule. We have already obtained evidence that hypermethylation has a disruptive effect on tRNA structure (Pillinger *et al.* 1969) and the work of Lindahl, Adams, Geroch & Fresco (1967) has shown that inactivation of the function of tRNA can be brought about by changes in the tertiary structure.

However, in attempting to find a relationship among the functions of tRNA, it would seem reasonable to propose that there may be a number of different mechanisms whereby a specific tRNA might carry out its functions. Perhaps there are several possible recognition mechanisms to relate an aminoacyl-tRNA synthetase to its specific tRNA even in one cell type, since evolutionary restraints on this interaction should be less demanding than, for example, on codon-anticodon recognition. If this were so, no single recognition mechanism would cover the aminoacyl-tRNA synthetase-tRNA interaction, nor could we predict that in all tRNA species the anticodon region was or was not part of these recognition systems.

Formation of polypeptide. (i) Poly-L-phenylalanine formation. The amino acid-accepting ability of phenylalanine tRNA is inhibited by 50% after hypermethylation. Such hypermethylated tRNA is still fully able to bind to ribosomes, yet its ability to form polypeptide is inhibited to an extent much greater than could be predicted from inhibition of acceptance alone. In this case, the presence of extra methyl groups in the molecule has an effect on the aminoacylation function and not on codon-anticodon recognition. In addition, however, a further specificity of the phenylalanine tRNA molecule must be sensitive to hypermethylation.

Most probably this inhibitory effect of hypermethylation relates to the inability of the tRNA to carry out some function during protein synthesis at the ribosome itself. We know that the tRNA will bind, at least in some fashion, to ribosomes in the presence of the correct triplet codon. However,

subsequent operations, e.g. transfer of the tRNA molecule from site to site on the ribosome or release of the tRNA from ribosome-binding, may be inhibited by hypermethylation. However this may be, it is clear that hypermethylation of tRNA can disrupt its activity in several distinct directions, either by alteration of a specific base or by a three-dimensional structural change.

(ii) Poly-L-proline and poly-L-lysine formation. Proline tRNA and lysine tRNA that had been hypermethylated to the extent of 7 mol % excess of methyl groups were able to accept amino acids at 50% and 80% of the efficiency of normal tRNA respectively (Pillinger *et al.* 1969). However, the same hypermethylation completely prevented the function of prolyl-tRNA in polypeptide synthesis and inhibited this function of lysyl-tRNA by 60%.

Clearly the effect of hypermethylation on these tRNA species is not restricted to their amino acid-acceptance ability and one or more of the additional functions of tRNA must be susceptible to hypermethylation, perhaps because of methylation at a specific region in the molecule or by general interference by the secondary and tertiary structure. It might be speculated, for example, that the formation of 7-methylguanine at the anticodon region of prolyl-tRNA (which may contain at least two guanine residues; Crick, 1966) could be responsible for its inactivation in polypyrrol synthesis. Proof of this point, however, requires establishment of the sequence at the anticodon site of the molecule and completion of the relevant binding experiments.

Weil, Befort, Rether & Ebel (1964) have shown that the transfer function of yeast lysine tRNA was less affected by hypermethylation than that of phenylalanine tRNA and have attributed this observation to alterations at the anticodon sites of these molecules.

It seems reasonable to conclude from these experiments and from earlier observations (Pillinger *et al.* 1969) that the introduction of extra methyl groups into a tRNA molecule has a profound effect on several of its functions. Borek (1969) has suggested that hypermethylation of tRNA might play an important role *in vivo* in the control of protein synthesis. Such control might operate in at least two ways: it might either inactivate one of the functions of a tRNA molecule and effectively

remove it from the cell, or alter its function such that it might accept a different amino acid, or, perhaps, recognize a new codon. The present results indicate that the first of these possibilities might be operational.

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