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

FORMATION OF AMINOACYL-TRANSFER-RIBONUCLEIC ACID

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(Received 28 April 1969)

1. The ability of chemically hypermethylated *Escherichia coli* B transfer RNA to accept 19 amino acids was studied and the results were compared with those obtained with a control sample of *E. coli* B transfer RNA incubated under similar conditions in the absence of methylating agent. 2. There is a marked decrease in the ability of the modified transfer RNA to accept amino acids in almost all instances. 3. The acceptance of cysteine appears to be unique in that it is enhanced in the hypermethylated transfer RNA. 4. More detailed studies on the kinetics of acceptance for six amino acids is presented, emphasizing the variation in response of the individual amino acids. 5. Increasing hypermethylation causes a progressive decrease in the amino acid acceptance. 6. The results are discussed in terms of methylation at functional sites within the transfer RNA and possible conformational alterations to the structure of the macromolecule.

The formation of aminoacyl-tRNA[†] represents the first stage in protein synthesis at which discrimination among amino acids takes place. Studies on the specificity of this reaction have shown that various degrees of interchangeability of the aminoacyl-tRNA synthetases and tRNA from different organisms can take place, depending on the amino acid and the species from which the tRNA and the enzymes are derived (Benzer & Weisblum, 1961).

One aspect of the structure of tRNA that makes each molecule separate and distinct from others is the pattern of methylation of bases superimposed on the primary sequence of nucleotides. It seems reasonable to suppose, in the absence of complete experimental data, that such methylation plays an important part in all the recognition functions associated with tRNA molecules.

Although the species specificity of tRNA methylation is now well recognized, the role of methylated bases in the biological function of tRNA has remained obscure despite efforts at its elucidation. Studies comparing amino acid acceptance in normal and under-methylated tRNA showed no detectable differences in this parameter between the two forms (Starr, 1963; Littauer, Muench, Berg, Gilbert & Spahr, 1963; Peterkovsky, Jesensky,

Bank & Mehler, 1964). However, in each of these studies, results were compromised as the methyl-deficient tRNA contained 50% of normally methylated RNA. More recently, homogeneous preparations of methyl-deficient tRNA have become available and it has been shown that the distribution of the methyl groups is a determinant factor in amino acid acceptance: tRNA devoid of methyl groups accepts amino acids at a fraction of the rate that its fully methylated counterpart does (Shugart, Novelli & Stulberg, 1968).

Supernumerary methyl groups can be introduced into tRNA by heterologous interactions, especially by enzyme extracts from tumour tissue; they can also be introduced by methylation with chemical methylating agents. We report here that base-specific hypermethylation of tRNA by dimethyl sulphate affects the rate and extent of acceptance of individual amino acids in a variety of cases.

MATERIALS AND METHODS

Preparation of aminoacyl-tRNA synthetases. The aminoacyl-tRNA synthetase enzymes (EC 6.1.1.-) were prepared essentially by the method of Nishimura & Novelli (1964).

E. coli B cells grown in 0.3% glucose-salts medium were harvested in the exponential phase at a concentration of 5×10^8 cells/ml. After being washed once with 0.15M NaCl, the cells were suspended in 10mM-tris-HCl buffer, pH 7.5, containing 10mM-magnesium acetate and 1mM-mercaptoethanol and ground in a mortar with 6.0g. of alumina

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[†] Abbreviation: tRNA, transfer RNA.

(Alcoa A-305). The volume was made up to 30 ml. before centrifugation at 20000g for 20 min. followed by 150000g for 90 min. The final supernatant was removed and dialysed against 2 l. of suspending buffer at 4° for 18 hr. The pH of the dialysed supernatant was adjusted to neutrality with 0.3 M-KOH, and 0.1 vol. of 10% (w/v) streptomycin was added. After the mixture had been stirred at 4° for 3 hr., the precipitate was removed by centrifugation at 10000g for 10 min. The supernatant was adjusted to pH 7.5 and solid $(\text{NH}_4)_2\text{SO}_4$ was added with stirring to give 50% saturation. The supernatant either from this preparation or from the precipitation with streptomycin was used as a source of synthetase enzymes after further dialysis: in either form the precipitate could be stored at -20° for several weeks.

Assay of amino acid acceptance. Amino acid acceptor activity was assayed with a reaction mixture containing 250 μg . of tRNA (6 E_{260} units measured at pH 12), 5 μmoles of magnesium acetate, 50 μmoles of tris-HCl buffer, pH 7.2, 0.5 μmole of ATP, 0.05 μmole of CTP and 5 μmoles of KCl in a volume of 0.25 ml. To this, 0.3 μC of the ^{14}C -labelled amino acid being studied was added in a solution containing 0.125 μmole of each of the 19 unlabelled amino acids, and 300 μg . of aminoacyl-tRNA synthetase preparation was added in a final volume of 0.5 ml. The tRNA was obtained from General Biochemicals Inc., Chagrin Falls, Ohio, U.S.A.

Incubation under standard assay conditions was at 37° for 10 min. At the end of this time 0.05 ml. portions of each reaction mixture were transferred in duplicate to filter-paper discs and submerged in cold 5% (w/v) trichloroacetic acid. The discs were washed twice in 5% trichloroacetic acid, then ethanol-ether (1:1, v/v) and ether, before being dried. The radioactivity on each disc was assayed in a low-background gas-flow counter.

Chemical methylation of tRNA. *E. coli* B tRNA (10 mg.) in 0.1 M-sodium phosphate buffer, pH 7.5 (2.0 ml.), was incubated at 25° with [^3H]dimethyl sulphate (80 μmoles). After 2 hr. of the incubation, 0.1 vol. of 20% (w/v) potassium acetate and 2 vol. of cold ethanol were added to precipitate the tRNA. The methylated tRNA was dissolved and reprecipitated twice more, taken into solution in 1.8 M-tris-HCl buffer, pH 8.0 (1 ml.), and incubated at 37° for 30 min. Finally the tRNA was precipitated and taken into solution to contain 60 E_{260} units/ml.

A similar sample of tRNA incubated at 25° in phosphate buffer in the absence of dimethyl sulphate but subjected to the same purification procedure served as a control for subsequent experiments.

To obtain a series of tRNA samples methylated to different extents, the incubation was repeated with a range of six concentrations of [^3H]dimethyl sulphate from 1 μmole to 32 μmoles /mg. of RNA. In each case the number of methyl groups introduced was calculated from the specific radioactivities of the tritiated RNA samples isolated and expressed as moles of methyl group/100 moles of nucleotide excess.

Measurement of extinction as a function of temperature. The effect of temperature on the extinction of methylated tRNA in solution was studied by using a Gilford recording spectrophotometer and a cuvette housing heated by circulating water. The tRNA was dissolved in 10 mM-tris-HCl buffer, pH 7, containing 1 mM-magnesium acetate and 0.15 M-NaCl. The temperature rise did not exceed 1.5°/min.

Measurement of sedimentation coefficients. The sedimenta-

tion coefficients of methylated tRNA samples were measured in the Spinco model E analytical ultracentrifuge. The tRNA was dissolved in 0.15 M-NaCl for this purpose.

RESULTS

The significance of any observations relating the effect of chemical modification to biological function is dependent on the demonstration of absence of gross destruction of the macromolecule during the modifying reaction. The results obtained for the sedimentation characteristics of methylated tRNA (7 moles % excess of methyl groups) and normal tRNA, obtained through the courtesy of Dr H. Rosenkranz, were found to be 3.65 and 3.45 s respectively. Limited removal of 3'-hydroxy-terminal nucleotides may have taken place and this would probably not be revealed by the above techniques. However, a comparison of the activity of tRNA treated as above in the absence of dimethyl sulphate with that of tRNA that has not been through the incubation procedure suggests that this cannot be a major factor. We conclude therefore that, under the conditions of alkylation selected, the methylated tRNA is essentially undegraded.

The results of studies on the effects of increasing hypermethylation on the 'melting' characteristics of tRNA are presented in Fig. 1. Since a heterogeneous population of tRNA species was exposed to the methylating agent the physical measurements are only a statistical summation of divergent effects on the individual RNA species. Addition of up to 4% excess of methyl groups has relatively little

Fig. 1. Ultraviolet absorption-temperature profiles for *E. coli* B tRNA in 10 mM-tris-HCl buffer, pH 7, containing 0.15 M-NaCl and 1 mM-magnesium acetate (Δ) and after methylation with dimethyl sulphate in which 3.0 ($\square$), 4.4 ($\circ$), 7.0 ($\blacktriangle$), 9.2 ($\blacksquare$), 10.6 ($\bullet$) and 13.0 ($\times$) moles % excess of methyl groups were introduced.

Table 1. *Percentage change in amino acid acceptance after methylation of E. coli B tRNA*

The reaction mixture, incubated at 37° for 10 min., contained 50 μ moles of tris-HCl buffer, pH 7.2, 5 μ moles of magnesium acetate, 5 μ moles of KCl, 0.5 μ mole of ATP, 0.1 μ mole of CTP, 150–250 μ g. of enzyme protein, 250 μ g. of 'stripped' tRNA, 0.125 μ mole of each of 18 amino acids and 0.300 μ C of one ¹⁴C-labelled amino acid. The final volume was 0.5 ml. The experiments presented involved the use of three separate enzyme and methylated tRNA preparations. Results are expressed as an average of at least three experiments $\pm$ s.e.m.

Change in amino acid acceptance after methylation (%)		Change in amino acid acceptance after methylation (%)	
Amino acid		Amino acid	
Ala	-48 $\pm$ 3	Lys	-22 $\pm$ 5
Arg	-63 $\pm$ 2	Met	-76 $\pm$ 3
Asp	-46 $\pm$ 5	Phe	-50 $\pm$ 2
Cys	+57 $\pm$ 23	Pro	-50 $\pm$ 1
Glu	-55 $\pm$ 3	Ser	-54 $\pm$ 4
Gln	-48 $\pm$ 4	Thr	-38 $\pm$ 3
Gly	-69 $\pm$ 3	Trp	-70 $\pm$ 10
His	-40 $\pm$ 9	Tyr	-34 $\pm$ 14
Ile	-39 $\pm$ 1	Val	-49 $\pm$ 1
Leu	-56 $\pm$ 4		

effect on the 'melting' profile of tRNA. From 4% excess to 13% excess there is a marked alteration in profile and the co-operative effect in 'melting out' organized regions characteristic of double-stranded molecules (Doty, Boedtker, Haselkorn & Litt, 1959) progressively disappears. In addition, as over-methylation increases, there is a corresponding decrease in hyperchromicity. This, again, suggests that the entropy of these molecules is increasing with successive over-methylations. It is reasonable to suppose that, at low extents of methylation, the bases modified occur primarily in regions that are not directly involved in base-pairing; these regions, as far as we can judge from current ideas on tRNA structure, would include looped-out regions generally considered to be active in the various recognition functions of tRNA. If, however, the initial methylation sites are in structured areas of the tRNA, then the gross physical features of the molecule do not appear to be affected by the addition of up to 3 extra methyl groups/mol.

The capacity of hypermethylated tRNA to accept amino acids is presented in Table 1, in which the results of three experiments with three separate enzyme and methylated tRNA preparations are reported. In each case the acceptance of the amino acid by the hypermethylated tRNA was compared with that by a control tRNA that had been incubated in the absence of the methylating agent and purified by a similar procedure. In almost every instance there is a significant decrease in amino acid acceptance. The exception to this is found with cysteine, where the ability of hypermethylated tRNA to accept the amino acid is apparently enhanced.

A more detailed study on the kinetics of accept-

ance for six amino acids confirmed the above results and emphasized the variation in response of individual amino acids to the hypermethylated tRNA (Fig. 2). For example, the acceptance of phenylalanine by unmodified *E. coli* tRNA rises steadily, reaching a plateau value by 20 min. For the hypermethylated tRNA the rate of uptake of labelled amino acid is noticeably lower, but the plateau value is achieved after a comparable time-interval. In the latter case, however, the acceptance of amino acid is only 50% of that by the unmodified control tRNA. The stability of the resulting aminoacyl-tRNA complex appears to be comparable in the two instances. Similar results were obtained for the formation of prolyl-tRNA.

For serine tRNA and alanine tRNA (Fig. 3) the initial rates of formation of the complexes were comparable for the first 3 min., after which the amount of amino acid accepted by unmodified tRNA species continued to increase steadily, reaching a maximum at about 10–15 min. The incorporation of labelled amino acid into the methylated tRNA species virtually ceased after 4 min., thereby accounting for the discrepancy of 50% in amino acid acceptance found during the standard assay at 10 min. The stability of the respective aminoacyl-tRNA species was apparently different in the two cases. Although the control alanyl-tRNA was subject to steady breakdown during the subsequent 50 min., the product from the methylated nucleic acid was stable over the same period. The breakdown of seryl-tRNA was more evident, such that by 80 min. there was little difference between the amount of radioactive amino acid remaining associated with the control tRNA and methylated tRNA.

Hypermethylated tRNA accepted about 25%

Fig. 2. Amino acid acceptance by normal and hypermethylated *E. coli* B cysteine tRNA (a), phenylalanine tRNA (b) and proline tRNA (c) as a function of time. The reaction mixture was identical with that described in Table 1. ●, Normal *E. coli* B tRNA; ○, hypermethylated *E. coli* B tRNA (7.0 methyl groups excess/mol.).

less lysine than did the control, and this decrease was consistent at times studied up to 90 min. There was apparently no difference in stability of the aminoacyl-tRNA species in this instance. As noted above, the formation of cysteinyl-tRNA appeared to be unique in that the methylated RNA accepted appreciably more cysteine than the *E. coli* RNA used as control.

The results of these kinetic studies illustrate the specificity of response with the individual amino acid, and this may account, in part, for the irregular and discordant values obtained previously under standard assay conditions (Weil, Belfort, Rether & Ebel, 1964; Zakharyan, Venkstern & Bayev, 1967).

The inhibition of amino acid acceptance with increasing hypermethylation of the tRNA is shown in Fig. 4. In each instance increasing the extent of methylation resulted in progressively less amino-

Fig. 3. Amino acid acceptance by normal and hypermethylated *E. coli* B serine tRNA (a), alanine tRNA (b) and lysine tRNA (c) as a function of time. The reaction mixture was identical with that described in Table 1. ●, Normal *E. coli* B tRNA; ○, hypermethylated *E. coli* B tRNA (7.0 methyl groups excess/mol.).

acyl-tRNA being formed. On purely chemical reasoning one could predict that the most reactive sites in the tRNA molecules would be methylated first, guanine sites being methylated before the adenine and cytosine residues react. If one assumes that methylation at a critical site, such as the anticodon region, the CCA terminal triplet or some other location in the molecule, is necessary to render the molecule incapable of forming the aminoacyl complex, it should be possible to achieve this critical extent of methylation in each molecule after which the formation of aminoacyl-tRNA would be unaffected no matter how many more

Fig. 4. Inhibition of amino acid acceptance as a function of the number of methyl groups introduced into *E. coli* B valine tRNA (a), lysine tRNA (b) and serine tRNA (c).

methyl groups were introduced. In view of the fact that some of the possible sites for alkylation already contain methyl groups from normal methylation processes *in vivo* and others are in unfavourable positions to receive the bulky methyl group by virtue of the secondary structure of tRNA, it is unlikely that an extra 13–15 methyl groups need to be introduced/molecule before complete inhibition of acceptance, as with serine tRNA in Fig. 4. This progressive inhibition could be interpreted, however, if the introduction of a methyl group into a tRNA molecule modified the secondary structure such that other sites within the molecule became more amenable to subsequent methylation than the initial site in another tRNA molecule. Such an effect is analogous to a first-order chemical reaction in which a rate-determining step influences the kinetics of the reaction. In other words, the population of tRNA species used in the acceptance assay is a heterogeneous one with respect to methylated bases; some molecules may have many more methyl groups than average whereas others have less or none at all. As the tRNA is exposed to increasing molar proportions of dimethyl sulphate during the chemical hypermethylation, the percentage of molecules containing the site or sites necessary for acceptance of amino acid becomes progressively less.

Studies of amino acid acceptance and the kinetics of reaction with a purified phenylalanine tRNA, kindly supplied by Dr G. D. Novelli, have yielded the same results as those obtained from studies with the mixed tRNA species obtained commercially. The presence of tRNA species specific for other amino acids does not affect the

results for individual tRNA species. No evidence was found for the misacceptance of other amino acids by a methylated sample of the purified phenylalanine tRNA.

DISCUSSION

The ultimate stage in the cellular synthesis of tRNA is enzymic methylation of the product of DNA transcription. If tRNA plays a part in cellular control mechanisms, as has been suggested (Kaneko & Doi, 1966; Kjellin-Straby & Phillips, 1968), then the specific numerical distribution and siting of the minor nucleotides along the RNA chain may represent a subtle means of modifying the structure of tRNA for some cognitive function.

At least three different functional attributes can be ascribed to tRNA: coding, recognition of or by aminoacyl-tRNA synthetase enzyme and the charging of the terminal adenosine. Our studies have shown that chemical hypermethylation affects both the rate and the extent of the charging of the terminal nucleoside. This alteration in the functional ability of the tRNA could be mediated at the anticodon region during recognition of the synthetase enzyme or at the acceptor site by the introduction of methyl groups at sites within the molecule particularly concerned with these reactions. A further possibility, that a conformational alteration consequent on methylation modifies one or more of these reactions, must also be considered.

Analysis of the bases methylated after reaction with dimethyl sulphate showed that the predominant site for alkylation was the 7-position of guanine. 1-Methyladenine was also formed, but only trace amounts of 3-methylcytosine were detected.

If the alteration in acceptor activity was directly attributable to modification of nucleotides in the anticodon region, it should be possible to correlate inhibition of acceptance with the base composition of the anticodon. There should, for example, be little or no change in lysine acceptance as the anticodon for this amino acid contains predominantly uridine, which is not methylated by dimethyl sulphate. The formation of lysyl-tRNA was, in fact, found to be inhibited least in hypermethylated tRNA. Conversely, the inhibition of acceptance should be greatest for proline tRNA, for which the anticodon contains predominantly guanosine. A decrease of 50% in the acceptance of proline by the methylated RNA was found. However, the formation of glycyl-tRNA and glutaminytRNA, which contain mainly cytidine and aridine in their anticodons, was inhibited more than that of prolyl-tRNA, whereas the formation of phenylalanyl-tRNA was inhibited to an extent similar to that of prolyl-tRNA, although adenosine predominates in its anticodon. Clearly the results

obtained cannot be interpreted convincingly on the basis that methylation at the anticodon alone is responsible for the present observations.

Very little is known about the functional sites in tRNA species concerned with the recognition of specific aminoacyl-tRNA synthetases. The pattern of species specificity that has emerged from the studies by Doctor & Mudd (1963) on cross-reactions between tRNA and aminoacyl-tRNA synthetases from different sources suggests that the site of interaction in tRNA varies from species to species. If one accepts the universality of the genetic code, it seems unlikely that the enzyme-recognition site is synonymous with the anticodon sequence. If the enzyme-recognition site is different from the anticodon site, the possibility arises that methylation of the molecule could disrupt the recognition ability specifically and could then be responsible for alteration in acceptance alone. A possible role for tRNA methylation *in vivo* would then exist and could represent a means whereby species specificity could be imparted to a structure originally derived by transcription of only a few bases. Support for the suggestion that the anticodon sequence and the enzyme-recognition site are discrete comes from studies of the effect of modification of tRNA by treatment with nitrous acid (Carbon, 1964) and irradiation of tRNA by u.v. light (Fukutome, Kawade & Morita, 1964).

However, work by Hayashi & Miura (1966), in which they caution against comparisons of species specificity without reference to possible differences in three-dimensional structures of the tRNA, suggests that in *E. coli* tRNA the recognition site is related to the anticodon sequence of tRNA. In support of this interpretation they quote the results of Penniston, Steward & Tucker (1964), showing that the rate of inactivation of the aminoacylation activity and the transfer activity for formaldehyde-modified tRNA were directly related, and work on modification of uracil residues by u.v. light (Zachau, 1964) and by hydroxylamine (Kisselev & Frolova, 1964; Cerna, Rychlik & Šorm, 1964). If the enzyme-recognition site and the anticodon are identical or even related, then it is unlikely that the recognition site is the primary locus for chemical methylation and is responsible for the inhibition of acceptance observed in methylated tRNA, by the same argument that was used above for the anticodon.

A recent finding by Thiebe & Zachau (1968) implies that a modified base adjacent to the anticodon may confer a unique conformational individuality on tRNA, and this may serve as a cognitive site.

Studies on several parameters, including hyperchromicity, X-ray diffraction and optical rotation, have shown that tRNA molecules assume con-

formations containing organized regions of helical structure. It is conceivable therefore that the specificity associated with the tRNA molecule could be generated merely by the presence of stabilized regions of secondary structure. The inadequacy of a hypothesis based on different conformations within a particular nucleotide sequence each having similar helical content and energy giving rise to a unique structure has been discussed by Fresco, Adams, Ascione, Henley & Lindahl (1966). By virtue of their partial helical structure, however, tRNA molecules do possess a potential for tertiary structure, in this case the unpaired bases forming loci of interactions such that the helical segments become spatially fixed. The change in chromatographic behaviour of methyl-deficient phenylalanine tRNA and the differential effects of Mg^{2+} concentration on coding properties of normal and methyl-deficient phenylalanine tRNA confirm the role of methylation in conserving secondary structure. Correlation between the ability of tRNA to be enzymically acylated with amino acid and loss of hyperchromicity during thermal denaturation of tRNA in dilute salt solution has shown that aminoacylation ceases when only 10% of the total hyperchromic shift has occurred.

Chemical hypermethylation of tRNA leads to loss of secondary structure (Fig. 1). The results we have obtained with tRNA populations that have been progressively over-methylated (Fig. 4) show that an approximately linear response of inhibition of acceptance is obtained. This type of response would not be incompatible with the hypothesis that the effect of over-methylation is generated via a general effect on secondary or tertiary structure in tRNA, which in turn is responsible for the specificity of amino acid acceptance.

The work reported in this paper was undertaken during the tenure of a Research Fellowship awarded to D.J.P. by the International Agency for Research on Cancer. We were also aided by a grant from the Damon Runyon Memorial Foundation and by grants from the National Institutes of Health and the Atomic Energy Commission. D.J.P. is on leave from the Paterson Laboratories, Christie Hospital and Holt Radium Institute, Manchester M20 9BX. J.H. is on leave from the Department of Biochemistry, University of Glasgow.

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