STUDIES ON INHIBITORS OF MAMMALIAN tRNA METHYLASES

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1. Introduction

Although the function of the methylated nucleosides in tRNA is not well understood numerous papers have suggested that methylation of tRNA may be of importance in cellular regulatory mechanisms since alterations in the activities of tRNA methylases assayed in vitro have been detected in many tissues undergoing changes in growth or differentiation under a variety of stimuli [1–10]. Recently several authors have reported that inhibitors of these enzymes are present in some tissues and that the changes in the activities of tRNA methylases may be brought about by alterations in the concentration of such inhibitors [8–10]. The nature of these naturally occurring inhibitors is not yet clearly defined and only limited experimental data on compounds inhibiting tRNA methylases of mammalian cells have been published [3, 11, 12]. The availability of a potent inhibitor of tRNA methylase activity may provide a useful means of investigating the role of methylation in the function of tRNA and the growth of the cell.

This paper describes the effects of a number of compounds related to *S*-adenosyl-L-methionine (AMe) on the activity of tRNA methylases isolated from rat liver and kidney. *S*-Adenosyl-L-homocysteine (AH) was found to be a potent inhibitor of methylation when present at concentrations of the same order as AMe and could have an effect on the methylation of nucleic acid in vivo.

2. Materials and methods

S-Adenosyl-L-[Me-^{14}C]-methionine (AMe-$^{14}CH_3$) was purchased from The Radiochemical Centre, Amersham, and purified and stored as previously described [13]. *S*-Adenosyl-L-ethionine (AEt) and Se-adenosyl-L-selenomethionine (SeAMe) were prepared from the amino acids by the action of ATP:L-methionine *S*-adenosyltransferase [13, 14]. *S*-Adenosyl-L-homocysteine was purchased from Boehringer Corp. (London) Ltd. L-Ethionine, L-methionine, L-selenomethionine and *S*-methyl-L-methionine were products of Sigma Chem. Co., London. *S*-Trifluoromethyl-L-homocysteine was obtained from Cyclo Chem. Corp., USA and tRNA^{fMet} from *E. coli* K 12 was a gift from Oak Ridge National Laboratory, N.I.H., USA.

Tissue extracts containing tRNA methylase were prepared from homogenates of rat liver and kidney and assayed as described by Pegg [15]. The assay medium contained 15 μmoles tris-HCl pH 8.8, 4 μmoles putrescine, 50 μg of tRNA from *E. coli* K 12 or 250 pmoles tRNA^{fMet}, 0.2–0.5 mg enzyme protein and AMe-$^{14}CH_3$ and the compounds tested as inhibitors in a total volume of 0.15 ml. After incubation at 37° for 15 min aliquots were removed and the methylation of tRNA determined [15].

3. Results

When incubated under the conditions described above and in the presence of 25 μM AMe-$^{14}CH_3$, enzymes isolated from rat liver and kidney catalysed the methylation of bacterial tRNA at a rate which was

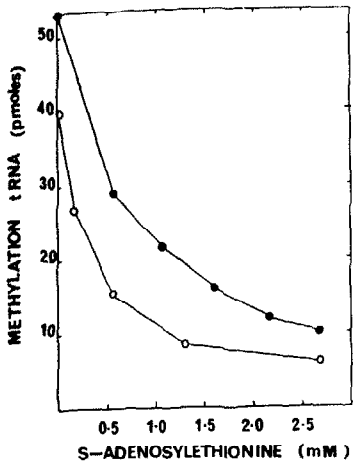

Fig. 1. Effect of AEt on methylation of tRNA by liver extracts. The assay medium contained 25 μM AMe-$^{14}CH_3$ (•) or 10 μM AMe-$^{14}CH_3$ (○), 50 μg tRNA and 0.3 mg of liver protein.

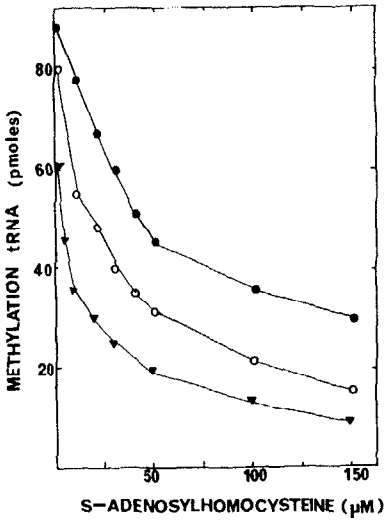

Fig. 2. Effect of AH on methylation of tRNA by liver extracts. The assay medium contained 22 μM (•), 9 μM (○) or 4.5 μM (▾) AMe-$^{14}CH_3$, 50 μg tRNA and 0.4 mg pf liver protein.

proportional to the amount of protein added and to the time of incubation for at least 30 min. L-Methionine (100 mM), L-ethionine (100 mM), adenosine (20 mM), *S*-methyl-L-methionine (10 mM) and *S*-trifluoromethyl-L-homocysteine (5 mM) had no effect on the methylation of tRNA when added to the assay medium at the concentrations shown. However, AEt, AH and SeAMe were effective in decreasing the transfer of radioactivity from AMe-$^{14}CH_3$ to tRNA.

As shown in fig. 1, AEt inhibited the methylation of tRNA but this sulphonium compound although closely related to AMe was not a particularly potent inhibitor since a concentration 30–40 times greater than that of the AMe-$^{14}CH_3$ present was required to achieve 50% inhibition. AH was a far more potent inhibitor of the methylation of tRNA by rat liver enzymes than AEt (fig. 2). 50% inhibition of the rate of methylation of tRNA was produced by concentrations of AH only 2–3 times greater than that of the AMe-$^{14}CH_3$. The inhibitory effect of AH was lessened at high concentrations of AMe-$^{14}CH_3$ suggesting that inhibition was of the competitive type. However, the data obtained by varying the concentration of AMe or AH and measuring the reaction velocity did not give simple graphical plots showing competitive inhibition. This finding is not unexpected since the methylation of unfractionated bacterial tRNA by a crude rat liver extract is made up of the actions of a number of enzymes producing a variety of methylated bases at a number of sites within the tRNA mixture added [1–10]. These enzymes may well have certain features in common and indeed all are inhibited in the presence

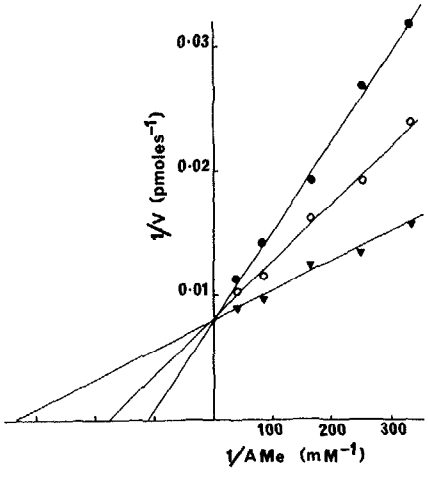

Fig. 3. Double reciprocal plot of methylation of tRNA against AMe-$^{14}CH_3$ concentration in the presence of 10 μM SeAMe (•), 7.5 μM AH (○) or no addition (▾). The assay medium contained 250 pmoles $tRNA^{fMet}$ and 0.6 mg of kidney protein.

of sufficiently high levels of AEt or AH but it is most unlikely that all have similar affinities for AMe and the inhibitors.

Purification of mammalian tRNA methylases has been hampered by the unstable nature of the enzymes and by the unavailability of the correct substrate (unmethylated mammalian tRNA) [1–3]. Since extensive purification of a particular tRNA methylase from a tissue extract cannot easily be accomplished without considerable loss of the original activity such a procedure is not suitable for estimations of any changes in the activity of the enzyme in developing or growing tissues. One approach which may prove useful in this respect is to use as a substrate for methylation a highly purified tRNA fraction which contains nucleotide sequences suitable for methylation by only one enzyme present in the tissue. Purified $tRNA^{fMet}$ from *E. coli* was methylated by an enzyme extract prepared from rat kidney and the total extent of methylation in the presence of excess enzyme was approximately equal to 1 pmole per pmole of $tRNA^{fMet}$ added. Preliminary evidence suggests that the product of this reaction is 1-methylguanine (Pegg, unpublished observations). The methylation of $tRNA^{fMet}$ by such kidney extracts was inhibited by AH and as shown in fig. 3 linear plots of the reciprocal of the AMe-$^{14}CH_3$ concentration with the reciprocal of the reaction velocity were obtained with this system. The K_m for AMe was 3 μM and the K_i for AH was 7 μM. The analogue of AMe in which the sulphur atom is replaced by selenium was also found to be a potent competitive inhibitor of the methylation of $tRNA^{fMet}$ (fig. 3). The K_i for SeAMe was found to be 5 μM.

Reaction of the amino group of the homocysteine moiety of AH with ethylchloroformate or *p*-nitrophenylchloroformate completely abolished the inhibitory activity. Similarly AMe in which the amino group of the methionine had been reacted with either of these compounds was no longer active as a methyl donor for tRNA methylases and had no effect on the rate of methylation of tRNA when AMe-$^{14}CH_3$ was available even when present at concentrations 100 times that of AMe-$^{14}CH_3$.

4. Discussion

The observations reported here utilising a crude preparation containing a number of tRNA methylases allow certain general conclusions concerning the requirements for inhibitors related to the methyl donor, AMe, to be made. These can be summarised as follows: (i) such compounds must have both an adenosyl and an amino acid moiety, (ii) the amino group of the amino acid must be free, (iii) the sulphonium centre is not obligatory but can be replaced by Se^+ or by the sulphur atom of AH and (iv) replacing the methyl group by an ethyl moiety has a much greater effect in reducing the activity of the inhibitor than removing the group completely (AH) or substituting the selenium atom for sulphur.

The three adenosyl derivatives found to affect the methylation of tRNA in vitro are all known to occur in rat liver under appropriate conditions and could therefore influence the methylation of nucleic acids in vivo. AEt accumulates in the livers of rats which had been treated with ethionine [16]. This compound can act as an ethyl donor in a number of enzymic reactions but, in general, the rate of reaction is considerably less than that of AMe [14, 16]. AEt is a substrate for some but not all of the tRNA methylases of rat liver and the rate of ethylation is less than 5% that of methylation [17]. It is possible that the ethylation of nucleic acids may be of importance in producing the physiological changes induced by ethionine administration [18] but the possible inhibition of methylation without consequent incorporation of ethyl groups at the nucleotide sites normally methylated should also be considered.

L-Selenomethionine is extremely toxic to rats and is known to be converted to the adenosyl derivative in the liver [18]. The selenium analogue of AMe has been found to be as active as AMe itself in reactions forming polyamines [14] and in certain methyl transfer reactions [18–20]. SeAMe may therefore be an active substrate for tRNA methylases. Further investigation of this possibility would require L-selenomethionine labelled in the methyl group. If SeAMe is able to replace AMe in this reaction the administration of L-selenomethionine would have little or no effect on the methylation of nucleic acids but if some or all of the tRNA methylases do not transfer methyl groups from SeAMe, administration of this amino acid

analogue may lead to the formation of 'under-methylated' tRNA.

Stekol has reported that *S*-trifluoromethyl-L-homocysteine was converted to *S*-adenosyl-L-trifluoromethyl-homocysteine by rat liver enzymes in vitro and in vivo and that after the administration of this amino acid to rats the transfer of methyl groups from AMe in vivo was decreased possibly by the inhibition of one or more transmethylation reactions by *S*-adenosyl-L-trifluoromethyl-homocysteine [18]. *S*-Trifluoromethyl-L-homocysteine had no effect on the methylation of tRNA in vitro and it was not possible to confirm the finding that this amino acid could be converted to an adenosyl derivative using Wistar rats from the strain bred in this Institute. Therefore the effects of this analogue of AMe on tRNA methylases could not be tested. Recently another report that *S*-trifluoromethyl-L-homocysteine could not be converted to the adenosyl derivative by an enzyme derived from rat liver has been published [21].

The competitive inhibition of tRNA methylases by AH which is a product of the reaction has also been found to occur with enzymes isolated from *E. coli* W [22]. The physiological significance of this inhibition is dependent on the relative levels of AMe and AH within the cell. The AH content of normal rat liver is 0.06 μmole/g wet tissue and is approximately equal to that of AMe [23]. It is therefore possible that in this tissue AH does exert some effect on the rate of methylation of tRNA and the AH present in undialysed extracts prepared from rat liver could have an inhibitory action on tRNA methylase activity measured in vitro. The AH present in liver homogenates could be at least partially responsible for the decreased tRNA methylase activity when liver extracts were added to tumour extracts [10], although, very recently, nicotinamide has been found to be an inhibitor of the methylation of tRNA and this compound may be responsible for all or part of the inhibitory activity present in liver extracts [11]. However, it should be noted that under normal metabolic conditions there is no pool of 'under-methylated' tRNA in rat liver [3] and hence the activity of tRNA methylases in vivo is sufficient to ensure complete methylation of tRNA. The demonstration that inhibitors of these enzymes are present in tissue extracts assayed in vitro [8–11] does not therefore prove that such inhibitors have physiological significance.

Acknowledgements

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