AMINOACYLATION OF ETHIONINE TO RAT LIVER tRNA^{Met} AND ITS INCORPORATION INTO PROTEIN

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1. Introduction

Ethionine is the carcinogenic ethyl analogue of methionine. The mechanism of its carcinogenicity is of particular interest since carcinogenic doses of ethionine do not result in alkylation of DNA [1]. The toxic and carcinogenic effects of ethionine can be completely prevented by elevated levels of methionine, therefore, ethionine apparently exerts its carcinogenic activity by interfering with some aspect of methionine metabolism. The ability of ethionine to replace methionine in protein synthesis has been studied extensively in procaryotic but not eucaryotic systems. Ethionine is aminoacylated to *Escherichia coli* tRNA^{Met} [2] and incorporated at normal methionine sites in bacterial protein [3]. In *E. coli*, ethionyl-tRNA$_f^{Met}$ is formylated by the bacterial transformylase enzyme [4] and formylethionyl-tRNA$_f^{Met}$ initiates polymethionine synthesis as directed by poly(AUG) [2]. Early reports of the incorporation of ethionine into eucaryotic protein relied on the precipitation of total protein from rat tissue [5]. While these studies may indicate that ethionine is incorporated into protein, they cannot rule out non-specific binding of ethionine to protein or ethylation of amino acid side chains.

This report examines the ability of ethionine to participate in eucaryotic protein synthesis. Using tRNA and aminoacyl-tRNA synthetases from the liver of a strain of rat susceptible to the hepatocarcinogenicity of ethionine, we demonstrate that ethionine is aminoacylated to tRNA^{Met} isoacceptors and is a competitive inhibitor of methionyl-tRNA synthetase. Further, we report similar rates of transfer of ethionine and methionine to protein from tRNA$_m^{Met}$ using a eucaryotic cell free protein synthesizing system.

2. Materials and methods

L-[^{3}H]Ethionine (100 mCi/mmol and 10 Ci/mmol), L-[^{14}C]methionine (256 Ci/mmol), L-[^{3}H]methionine (80 Ci/mmol, diluted to 10 Ci/mmol) and Aquasol and Econofluor scintillation solutions were purchased from New England Nuclear Corp. L-[^{14}C]Leucine (354 mCi/mmol) was purchased from the Radiochemical Centre, Amersham, and diluted to 38 mCi/mmol. L-Ethionine was obtained from Sigma Chemical Co. The in vitro translation components including wheat germ extract and rabbit globin mRNA were purchased from Bethesda Res. Labs. Micrococcal nuclease was obtained from P. L. Biochemicals.

Transfer RNA and aminoacyl-tRNA synthetases were prepared from the livers of Fisher 344 rats (7–8 weeks old male). Transfer RNA was isolated from 300 g tissue as in [6] and further purified on a DEAE-cellulose column [7]. Aminoacyl-tRNA synthetases were prepared from 50 g rat liver or from rabbit reticulocyte lysates under conditions which yielded all aminoacyl-tRNA synthetases active and free from endogenous amino acids [8,9]. [^{3}H]Ethionyl- and [^{14}C]methionyl-tRNA were prepared from bulk rat liver tRNA as in fig.1.

Purification of tRNA$_f^{Met}$ and tRNA$_m^{Met}$ was carried out as follows: bulk rat liver tRNA (200 A_{260} units) was fractionated on a RPC-5 column (1.3 × 29 cm) [10] in a 0.4–0.6 M linear NaCl gradient. Aliquots of the resulting fractions were aminoacylated with [^{3}H]-methionine [6,9]. Fractions containing tRNA$_f^{Met}$ and the tRNA$_m^{Met}$ isoacceptor which eluted last from the RPC-5 column were pooled separately and rechromatographed on a RPC-5 column as described [8,9].

Reaction mixtures for preparing [^{3}H]ethionyl- and [^{3}H]methionyl-tRNA$_m^{Met}$ contained excess levels of a

highly stable preparation of rabbit reticulocyte aminoacyl-tRNA synthetases, limiting levels of $tRNA_m^{Met}$ (0.03 A_{260} unit of tRNA/25 μl) and other components [9] with the exception that potassium phosphate buffer (pH 7.4) was used rather than Tris–HCl. Reaction mixtures were incubated at 37°C for 1 h; the resulting aminoacyl-$tRNA_m^{Met}$ were isolated and prepared for use in protein synthesis as in [6,8,11]. These conditions resulted in equivalent aminoacylation of [^{3}H]ethionine and [^{3}H]methionine to $tRNA_m^{Met}$ and were established from pilot studies.

Commercial wheat germ extract was treated with micrococcal nuclease to reduce endogenous mRNA [12] and protein synthesis subsequently carried out as given [13]. Reaction mixtures contained [^{3}H]-methionyl- or [^{3}H]ethionyl-$tRNA_m^{Met}$ (0.57 A_{260} units/100 μl), [^{14}C]leucine and excess [^{12}C]methionine (3.3×10^{-3} M). Transfer of [^{3}H]methionine or [^{3}H]ethionine to protein and deacylation of [^{3}H]-methionyl- and [^{3}H]ethionyl-$tRNA_m^{Met}$ were monitored as in [11] in the presence and absence of 0.5 μg globin mRNA/100 μl.

3. Results

The RPC-5 chromatographic profiles of [^{14}C]-methionyl- and [^{3}H]ethionyl-$tRNA^{Met}$ are shown in fig.1. The profiles are similar both in the number of isoacceptors aminoacylated and in their relative abundance. Isoacceptors of ethionyl-$tRNA^{Met}$ elute later from the column than the corresponding isoacceptors of methionyl-$tRNA^{Met}$. This greater affinity of ethionyl-$tRNA^{Met}$ may be due to a conformational change in the tRNA induced by the presence of the larger ethionine molecule. Although the elution profiles represented in fig.1 were obtained with [^{3}H]ethionyl- and [^{14}C]methionyl-$tRNA^{Met}$ which were prepared in the absence of 19 [^{12}C]amino acids (minus methionine), identical profiles were obtained when [^{3}H]-ethionyl- and [^{14}C]methionyl-$tRNA^{Met}$ were prepared in the presence of other amino acids.

The kinetic data for the attachment of methionine to purified $tRNA_f^{Met}$ and $tRNA_m^{Met}$ are given in table 1. The K_m-values for the attachment of methionine to $tRNA_f^{Met}$ and $tRNA_m^{Met}$ are similar. Ethionine was found to be a competitive inhibitor of methionyl-tRNA synthetase with K_i-values similar for both species of tRNA. K_m and K_i-values are comparable to those reported for *E. coli* [2]. The V_{max} for the formation of methionyl-$tRNA_m^{Met}$ is greater than for the formation of methionyl-$tRNA_f^{Met}$.

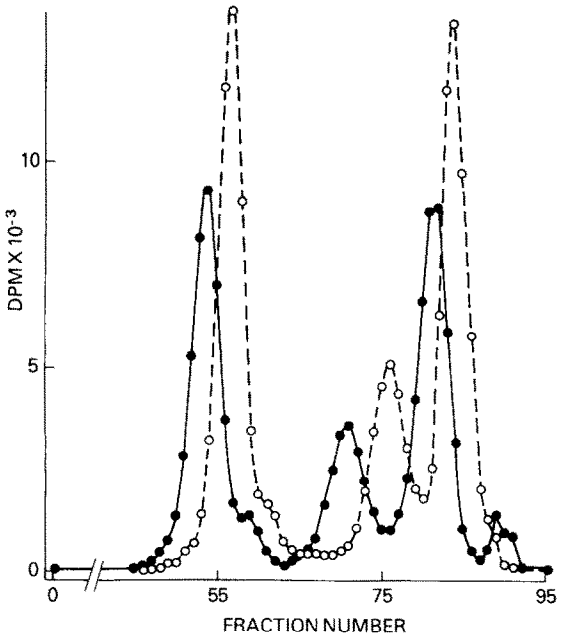

Fig.1. Chromatographic profiles of (•) [^{14}C]methionyl- and (○) [^{3}H]ethionyl-$tRNA^{Met}$. Bulk rat liver tRNA was aminoacylated with [^{14}C]methionine (256 mCi/mmol) and [^{3}H]ethionine (100 mCi/mmol) as in [6] in the absence of other amino acids. The reaction mixtures, which contained excess levels of freshly prepared rat liver aminoacyl-tRNA synthetases, were incubated at 37°C for 25 min. Following removal of the protein with phenol, the resulting aminoacyl-$tRNA^{Met}$ were precipitated, collected, pooled, applied to a RPC-5 column (1.3 × 29 cm) and chromatographed in a linear gradient of 0.4–0.6 M NaCl [8,11]. Fractions were collected directly into scintillation vials, Aquasol added and each counted in a Beckman scintillation counter. Counting efficiencies were determined using a series of quenched ^{3}H and ^{14}C standards and leakage of ^{14}C was determined from ^{14}C standards.

[^{3}H]Methionyl- and [^{3}H]ethionyl-$tRNA_m^{Met}$ were incubated with wheat germ extract in the presence and absence of globin mRNA (fig.2). [^{3}H]Methionine and [^{3}H]ethionine were transferred to protein, in response to globin mRNA, at similar rates. Rates of deacylation of [^{3}H]methionyl- and [^{3}H]ethionyl-$tRNA_m^{Met}$ were also very similar (not shown). [^{14}C]Leucine was included in the reaction mixtures to monitor the rate of protein synthesis (see inset in fig.2). [^{14}C]Leucine was incorporated into protein at equivalent rates in the presence of [^{3}H]methionyl- or [^{3}H]ethionyl-$tRNA_m^{Met}$ demonstrating ethionyl-$tRNA_m^{Met}$ did not interfere with overall protein synthesis.

Table 1
Kinetic data for the attachment of methionine to $tRNA^{Met}$ and its inhibition by ethionine

tRNA	K_m (μM)	K_i (mM)	V_{max} ($tRNA_m^{Met}$) / V_{max} ($tRNA_f^{Met}$)
$tRNA_f^{Met}$	1.1	0.17	3.6
$tRNA_m^{Met}$	2.4	0.27	

Reaction mixtures contained in a total volume of 25 μl excess levels of freshly prepared rat liver aminoacyl-tRNA synthetases and purified rat liver $tRNA_f^{Met}$ or $tRNA_m^{Met}$, [^{3}H]methionine (4×10^{-7}–2.8×10^{-5} M), other components [6] and [^{12}C]ethionine (2×10^{-4} M) where indicated. The reaction was terminated after 1 min at 37°C by the addition of 0.5 ml cold water followed by 7 ml 5% trichloroacetic acid. The resulting precipitates were collected on nitrocellulose filters, dried and counted in a Beckman liquid scintillation counter. K_m and K_i values were calculated from Lineweaver-Burk plots [14]

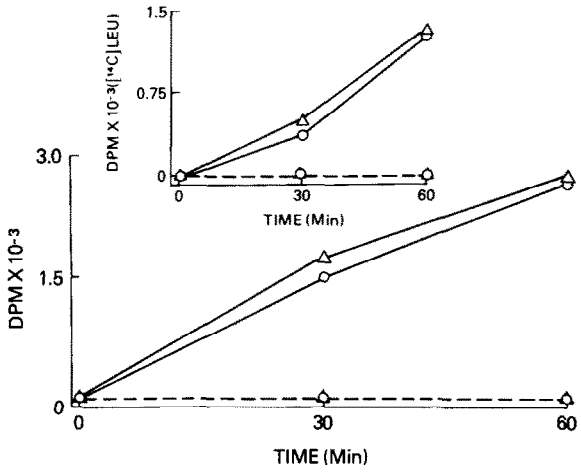

Fig.2. Utilization of [^{3}H]methionyl- and [^{3}H]ethionyl-$tRNA_m^{Met}$ in protein synthesis. [^{3}H]Methionyl- and [^{3}H]-ethionyl-$tRNA_m^{Met}$ (both at 10 Ci/mmol) were prepared as described and incubated in wheat germ extracts in the presence (——) and absence (– – –) of globin mRNA. The inset shows the rate of incorporation of [^{14}C]leucine into protein in response to globin mRNA in the presence of [^{3}H]methionyl- (△) and [^{3}H]ethionyl-$tRNA_m^{Met}$ (○). Precipitates at each time point were obtained as in [11], collected on nitrocellulose filters, dried, Econofluor added and each counted in a Beckman scintillation counter. Leakage of ^{14}C and counting efficiencies in precipitates, containing wheat germ extract, were determined from: (1) ^{3}H and ^{14}C standards were spotted on nitrocellulose filters which had been treated with trichloroacetic acid, dried and counted as above; (2) aliquots of [^{3}H]- and [^{14}C]aminoacyl-tRNAs containing levels of counts similar to those of the experimental samples were precipitated with trichloroacetic acid in the presence and absence of wheat germ extract, collected and counted.

4. Discussion

These studies show that ethionine is aminoacylated to initiator and internal methionine isoacceptors of rat liver by mammalian methionyl-tRNA synthetases. The same RPC-5 chromatographic profile of [^{3}H]ethionyl-$tRNA^{Met}$ is observed whether it is prepared in the presence or absence of 19 [^{12}C]amino acids (minus methionine). These data suggest ethionine is recognized only by methionyl-tRNA synthetase. Ethionyl-$tRNA^{Met}$ seems to manifest greater hydrophobicity than methionyl-$tRNA^{Met}$ as shown by its elution at a higher [NaCl] during RPC-5 chromatography. Kinetic studies with $tRNA_f^{Met}$ and $tRNA_m^{Met}$ show ethionine is a competitive inhibitor of methionyl-tRNA synthetase. Furthermore, ethionine and methionine are transferred to protein from $tRNA_m^{Met}$ at comparable rates in a eucaryotic cell-free protein synthesizing system programmed with globin mRNA.

Reported effects of ethionine on cellular macromolecules are ethylation and hypomethylation. Ethylation of DNA occurs only at levels of ethionine far in excess of carcinogenic doses and then at the N^7 position of guanine [15]. Ethionine does not produce alkylation of DNA at the O^6 position of guanine as do many carcinogenic, low M_r alkylating agents [16]. Hypomethylation of DNA has been reported in response to ethionine administration [17], but whether hypomethylation might be responsible for an altered gene expression relevant to carcinogenesis remains to be determined. Transfer RNA is more sensitive to ethionine administration than DNA. A rat liver lysyl-tRNA isoacceptor has been reported to be a major target of ethylation [18]. Hypomethylation of tRNA [19,20], as well as rRNA [21,22] and mRNA [23], following ethionine feeding has also been reported.

Although ethylation or hypomethylation of DNA or RNA provide a possible mechanism for the carcinogenicity of ethionine, the replacement of methionine by ethionine in protein synthesis with the subsequent production of non-functioning or mal-functioning proteins may be more significant. The synthesis of regulatory proteins with altered biological activity

might result in a selective growth advantage of affected cells. A mechanism whereby such an advantage could result in a permanent, heritable trait has been described [24].

We are examining the ability of ethionyl-$tRNA_f^{Met}$ to initiate protein synthesis and of the aminopeptidase to recognize and cleave ethionine from the N-terminal position. In addition, the ability of other amino acid analogues to transform cells in culture or produce tumors in animals is under investigation.

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