PROTEIN SYNTHESIS IN RABBIT RETICULOCYTES: CONTROL OF PROTEIN SYNTHESIS INITIATION BY A Met-tRNA$_f^{Met}$ DEACYLASE AND PEPTIDE CHAIN INITIATION FACTORS

N. K. Gupta and R. J. Aerni
Department of Chemistry
The University of Nebraska
Lincoln, Nebraska 68508

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Summary

The 0.5M KCl wash of rabbit reticulocyte ribosomes (I fraction) catalyzes the deacylation of Met-tRNA$_f^{Met}$. Upon DEAE-cellulose column chromatography, the deacylase activity elutes with the 0.1M KCl wash of the column (f1) and is well-resolved from the peptide chain initiation factors (1-3). The deacylase activity is specific for Met-tRNA$_f^{Met}$ (retic., E. coli). Other aminoacyl tRNAs tested including fMet-tRNA$_f^{Met}$ (retic., E. coli), Phe-tRNA (E. coli), Val-tRNA (retic.), and Arg-tRNA (retic.) are completely resistant to the action of the deacylase. In the presence of the peptide chain initiation factor (IF1) and GTP, retic. Met-tRNA$_f^{Met}$ forms the initiation complex Met-tRNA$_f^{Met}$:IF1:GTP (2), and in this ternary complex Met-tRNA$_f^{Met}$ is not degraded by the deacylase. E. coli Met-tRNA$_f^{Met}$ binds to IF1 independent of GTP, and in this complex, this Met-tRNA$_f^{Met}$ is degraded by the deacylase.

Prior incubation of f1 with Met-tRNA$_f^{Met}$ (retic.) strongly inhibited protein synthesis initiation, presumably due to deacylation of the initiator tRNA. This inhibition by f1 was completely prevented when Met-tRNA$_f^{Met}$ (retic.) was pre-incubated with peptide chain initiation factors.

Several laboratories have reported the presence of protein synthesis initiation inhibitors in rabbit reticulocytes (4-12). An inhibitor is formed during incubation of the post ribosomal supernatant (4) or lysate (5) at 34°. The formation of this inhibitor can be prevented by the addition of hemin (6-11) and the inhibitory activity of the preformed inhibitor can be overcome by the addition of the 0.5M KCl wash of reticulocyte ribosomes (I fraction) (7). The presence of an inhibitor in the crude I fraction which

inhibits the formation of the ternary complex Met-$tRNA_f^{Met}$:Initiation Factor: GTP (2, 12-13), has been reported by Dettman and Stanley (12).

In this communication, we present evidence for the presence of a Met-$tRNA_f^{Met}$ deacylase activity in the crude I fraction. Upon DEAE-cellulose chromatography, the deacylase activity elutes with the 0.1M KCl wash of the column (f1) and is separated from the peptide chain initiation factors. The deacylase activity is specific for Met-$tRNA_f^{Met}$ but does not degrade Met-$tRNA_f^{Met}$ in the Met-$tRNA_f^{Met}$:Initiation Factor:GTP initiation complex. The f1 fraction is strongly inhibitory to protein synthesis initiation, presumably due to deacylation of the initiator tRNA. By prior incubation of Met-$tRNA_f^{Met}$ with initiation factors, this inhibition due to f1 is overcome.

The presence of a Met-$tRNA_f^{Met}$ deacylase activity in reticulocyte ribosomes has been reported previously from this laboratory (2), and also by Morrisey and Hardesty (14).

Materials and Methods

The preparation of the 0.5M KCl wash of reticulocyte ribosomes (I fraction) was the same as has been described previously (3). The I fraction was further purified by passage through a DEAE-cellulose column (1). The Met-$tRNA_f^{Met}$ deacylase activity was not adsorbed on the column and was eluted with the 0.1M KCl wash (fraction 1 (f1)). A partially purified mixture of the peptide chain initiation factors (I fraction) used in this study was obtained by further eluting the DEAE-cellulose column with 0.3M KCl. All the enzyme preparations were thoroughly dialyzed against a buffer containing 5mM Tris-HCl pH 7.5, 100mM potassium chloride, 1mM dithiothreitol and 50μM EDTA.

Other materials and methods were the same as previously described (1-3, 15). Pure *E. coli* $tRNA_f^{Met}$ used in this study was a generous gift of Dr. G. David Novelli, Oak Ridge National Laboratory, Oak Ridge, Tennessee.

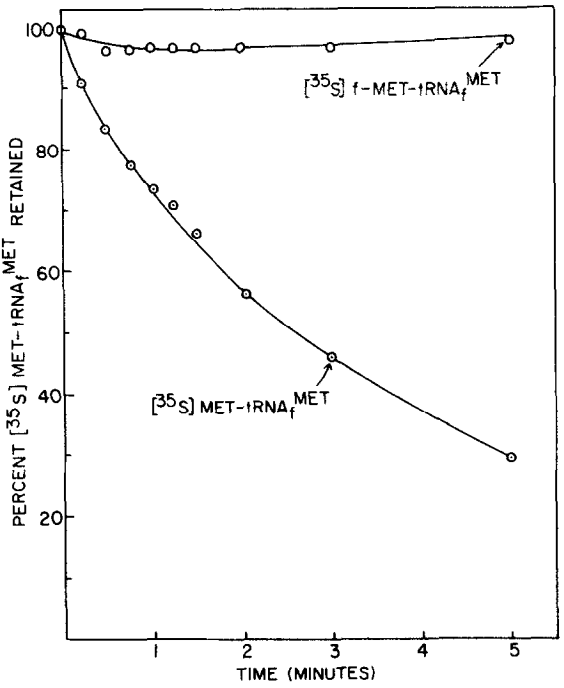

Figure 1. Kinetics of deacylation of [^{35}S]Met-tRNA$_f^{Met}$ (retic.) and [^{35}S]fMet-tRNA$_f^{Met}$ (retic.) by f1. Standard incubation mixtures contained (in a total volume of 0.3ml) the following: 20mM Tris-HCl (pH 7.5), 100mM potassium chloride, 2mM dithiothreitol, 10 pmoles [^{35}S]Met-tRNA$_f^{Met}$ (retic.) (18,000-20,000 cpm per pmole) or 15 pmoles [^{35}S]fMet-tRNA$_f^{Met}$ (retic.) and 160µg f1 protein. The reaction mixtures were incubated at 37°. At the indicated times, a 0.025 ml aliquot was spotted on filter paper disc (Whatman No. 3). The filter paper discs were then washed three times with cold 5% trichloroacetic acid, once with ethanol:ether (1:1) and once with ether. The filter papers were then dried and counted for radioactivity.

Results

The Met-tRNA$_f^{Met}$ deacylase activity was assayed using DEAE-cellulose purified f1 fraction. The result of a typical experiment is shown in Fig. 1. Approximately 70 percent of the input Met-tRNA$_f^{Met}$ was deacylated during the 5 minute incubation period. The deacylation reaction was dependent on the addition of f1; non-enzymic deacylation under the incubation condition was 8 percent of the input Met-tRNA$_f^{Met}$. The deacylase activity is specific for Met-tRNA$_f^{Met}$ and both retic. and E. coli Met-tRNA$_f^{Met}$ tested, were deacylated by the enzyme. Other amino acyl tRNAs tested, including

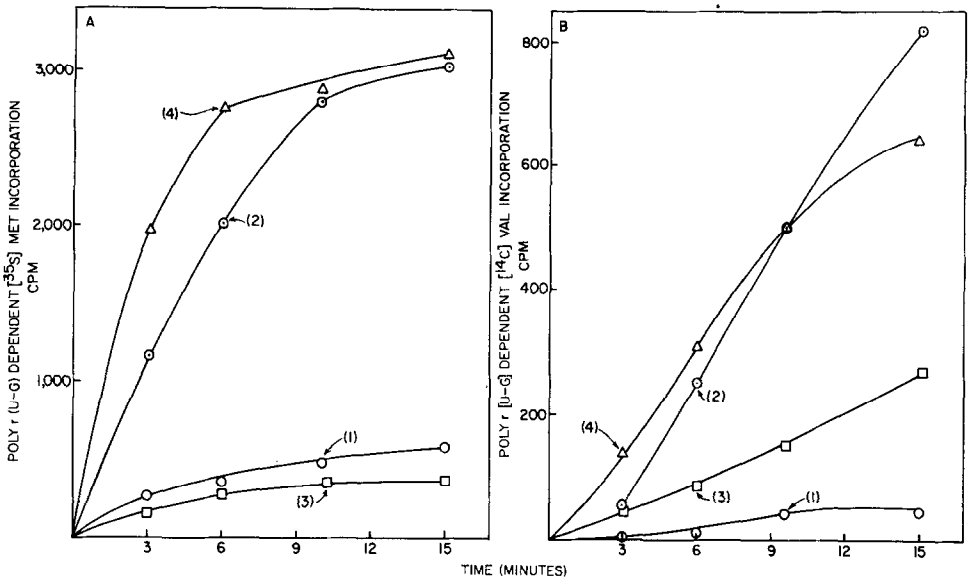

Figure 2. Effects of the addition of I fraction and f1 on poly r(U-G) directed amino acid incorporation.

A: [^{35}S]Methionine transfer from [^{35}S]Met-tRNA$_f^{Met}$ (retic.). Standard incubation conditions (in a total volume of 0.075 ml) were used as described previously (1, 3). Curve 1, complete reaction mixture minus I fraction and f1. Curve 2, complete reaction mixture with 30µg I fraction minus f1. Curve 3, [^{35}S]Met-tRNA$_f^{Met}$ was preincubated at 37° for 10 minutes with 80µg f1 and the reaction was started by the addition of I fraction and ribosomes. Curve 4, [^{35}S]Met-tRNA$_f^{Met}$ was preincubated at 37° with 30µg I fraction for 10 minutes and the reaction was started by the addition of 80µg f1 and ribosomes. At the indicated times, 0.05 ml aliquots of the reaction mixtures were assayed for hot trichloroacetic acid precipitable radioactivity by the filter paper disc assay method (1, 3).

B: [^{14}C] Valine incorporation: The reaction conditions were the same as above, except that the incubation mixture contained 10 nmoles of unlabelled cysteine, 0.5 nmoles [^{14}C] valine (350 cpm per pmole), and precharged unlabelled Met-tRNA$_f^{Met}$ present in a mixture of crude retic. tRNA (0.2 A_{260} unit per 0.075 ml reaction mixture).

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fMet-tRNA$_f^{Met}$ (retic., E. coli), Phe-tRNA (E. coli), Val-tRNA (retic.), and Arg-tRNA (retic.), were fully resistant to the action of the deacylase.

The enzymic cleavage products are methionine and deacylated tRNA$_f^{Met}$. Methionine liberated by enzymic deacylation was characterized by paper

TABLE I

Effects of the Addition of I and f1 on [^{35}S]Met-tRNA$_f^{Met}$ Binding and Deacylation

Experiment	[^{35}S]Met-tRNA$_f^{Met}$ Retained cpm/0.075 ml Incubation Mixture			[^{35}S]Met-tRNA$_f^{Met}$ Bound to Millipore Filter cpm/0.075 ml Incubation Mixture		
	-I+GTP	+I-GTP	+I+GTP	-I+GTP	+I-GTP	+I+GTP
A. Retic. [^{35}S]Met-tRNA$_f^{Met}$						
-f1	41,250	37,720	44,250	240	5,030	28,640
+f1, added after 5 min. incubation	2,100	5,250	28,500	1,360	3,970	23,850
B. E. coli [^{35}S]Met-tRNA$_f^{Met}$						
-f1	49,700	40,350	43,720	450	12,550	13,290
+f1, added after 5 min. incubation	1,350	2,020	2,320	840	1,530	1,180

Standard incubation conditions as described in Fig. 1 were used. The total reaction mixture was 0.075 ml. Where indicated, 0.2mM GTP and 30µg I fraction were added. The reaction mixtures were incubated at 37° for 5 minutes, at which time 40µg f1 was added in duplicate tubes. All the reaction mixtures were then incubated for another 5 minutes. Aliquots of the reaction mixtures were then assayed for total Met-tRNA$_f^{Met}$ retained, as described in Fig. 1, and for Met-tRNA$_f^{Met}$ binding to the initiation factor by the Millipore filtration technique as described previously (2).

chromatography. The tRNA$_f^{Met}$ liberated by the deacylation reaction was active in reinitiation of protein synthesis (Fig. 2B, Curve 3).

The results presented in Table I describes the effects of the I fraction and GTP on f1 catalyzed deacylation of Met-tRNA$_f^{Met}$. It has been previously reported that eukaryotic Met-tRNA$_f^{Met}$ forms an initiation complex with a eukaryotic initiation factor in the presence of GTP (2-3, 12-13). The Met-tRNA$_f^{Met}$:Initiation Factor:GTP complex is quantitatively retained on

Millipore filter and is assayed accordingly. In addition to retic. Met-$tRNA_f^{Met}$ and fMet-$tRNA_f^{Met}$, E. coli Met-$tRNA_f^{Met}$ and fMet-$tRNA_f^{Met}$ were also bound to the initiation factor but did not require GTP for such binding. The characteristics of Met-$tRNA_f^{Met}$ (retic., and E. coli) binding to the initiation factor is shown in Table 1, right hand columns. Approximately, 60 percent of the input retic. Met-$tRNA_f^{Met}$ was bound to the initiation factor in the presence of GTP. Under similar incubation conditions, 20-25 percent of the input E. coli Met-$tRNA_f^{Met}$ was bound to the initiation factor and this binding did not require GTP. Under the incubation conditions, the Met-$tRNA_f^{Met}$ binding to the initiation factor was complete within 5 minutes. In duplicate tubes, f1 was added after incubation of Met-$tRNA_f^{Met}$ with the initiation factor for 5 minutes and all the tubes were then further incubated for another 5 minutes. Aliquots of the incubation mixtures were then assayed for Met-$tRNA_f^{Met}$ binding to the initiation factor (Table 1, right hand columns) and also for deacylation of Met-$tRNA_f^{Met}$ (Table 1, left hand columns).

In the presence of added f1, there was approximately a 16 percent decrease in the amount of retic. Met-$tRNA_f^{Met}$ bound to the initiation factor in the presence of GTP, although the amount of Met-$tRNA_f^{Met}$ bound in the absence of GTP decreased more than 40 percent (after subtraction of background binding due to f1 in the absence of I fraction). Analysis for intact Met-$tRNA_f^{Met}$ by cold trichloroacetic acid precipitable radioactivity using similar aliquots indicated that in the absence of initiation factors and GTP, f1 degraded 95 percent of the input Met-$tRNA_f^{Met}$ (left hand columns); 85 percent of the input Met-$tRNA_f^{Met}$ was degraded when initiation factors were added in the absence of GTP but less than 40 percent of the input Met-$tRNA_f^{Met}$ was degraded when initiation factors and GTP both were added. The amount of Met-$tRNA_f^{Met}$ protected by the initiation factor and GTP was nearly equal to the amount bound to the initiation factor as assayed by the Millipore filtration technique.

In contrast to retic. Met-tRNA$_f^{Met}$, the binding of E. coli Met-tRNA$_f^{Met}$ to the initiation factor, did not protect the Met-tRNA$_f^{Met}$ from deacylation by f1. This is shown by both assays (Table I): Met-tRNA$_f^{Met}$ binding to the initiation factor (right hand columns, lower lines) and assay for intact Met-tRNA$_f^{Met}$ (left hand columns, lower lines).

The results presented in Fig. 2 describe the effects of the addition of f1 on protein synthesis initiation as studied using a reconstituted system with poly r(U-G) messenger and I fraction (1). The left hand figure (Fig. 2A) describes the transfer of methionine from [^{35}S]Met-tRNA$_f^{Met}$ into the terminal positions of the newly synthesized polypeptides and the right hand figure (Fig. 2B) describes overall protein synthesis as measured by the incorporation of [^{14}C] valine into the internal positions of the polypeptides. As reported previously (1), at 3mM Mg^{++}, the methionine transfer reaction in this reconstituted system is dependent on the addition of the I fraction (curves 1 and 2, Fig. 2A). In the experiment described in curve 3, Fig. 2A, [^{35}S]Met-tRNA$_f^{Met}$ was preincubated for 10 minutes with f1 in the protein synthesizing system and the reaction was started by the addition of I fraction and ribosomes. Complete loss of radioactivity transfer was observed, presumably due to deacylation of [^{35}S]Met-tRNA$_f^{Met}$ and dilution of [^{35}S] labelled methionine with excess unlabelled methionine added to the incubation mixture. In the experiment described in curve 4, Fig. 2A, [^{35}S]Met-tRNA$_f^{Met}$ was preincubated with the I fraction for 10 minutes in the protein synthesizing system, and the reaction was started by the addition of f1 and ribosomes. The methionine transfer activity in this experiment was completely protected and the initial rate of the methionine transfer reaction was significantly faster than the control experiment (curve 2, Fig. 2A). These results suggest that the Met-tRNA$_f^{Met}$:Initiation Factor:GTP is an active initiation complex, and Met-tRNA$_f^{Met}$ in this complex is not degraded by f1.

The results presented in Fig. 2B are essentially similar to those described in Fig. 2A. The incorporation of [^{14}C] valine in this system

was dependent on the addition of the I fraction and unlabelled methionine (Curves 1 and 2, Fig. 2B). Again, prior incubation of unlabelled Met-$tRNA_f^{Met}$ with f1 significantly inhibited [^{14}C] valine incorporation during the initial minutes (curve 3, Fig. 2B) and preincubation of unlabelled Met-$tRNA_f^{Met}$ with the I fraction prevented the inhibitory activity of f1 (curve 4, Fig. 2B). However, as shown in curve 3, Fig. 2B, the inhibition due to f1 was mostly overcome during the latter period of incubation. This observation is consistent with the suggestion that deacylated $tRNA_f^{Met}$ becomes re-acylated and in the presence of initiation factor forms the Met-$tRNA_f^{Met}$:Initiation Factor:GTP complex which is stable to deacylase activity.

Discussion

Although f1 actively deacylates Met-$tRNA_f^{Met}$ and thus causes inhibition of protein synthesis initiation in the cell-free system, the physiological role of this deacylase activity is not clear. The deacylase does not degrade the active initiation complex, Met-$tRNA_f^{Met}$ (retic.):Initiation Factor:GTP, but deacylates the complex of E. coli Met-$tRNA_f^{Met}$ with the initiation factor and possibly also the inactive initiation complex of retic. Met-$tRNA_f^{Met}$ formed in the absence of GTP. Our previous studies (15) showed that E. coli Met-$tRNA_f^{Met}$ is not recognized as an initiator tRNA in reticulocyte protein synthesis. The deacylase activity thus possibly monitors for the active initiation complex and frees Met-$tRNA_f^{Met}$ from inactive complexes with other proteins. Apparently, in the presence of excess initiation factor, protein synthesis initiation proceeds even in the presence of f1; deacylated $tRNA_f^{Met}$ is reacylated and is used for reinitiation (Fig. 2B, curve 3).

It is not clear how the inhibition of protein synthesis initiation observed in this work is related to the protein synthesis inhibition described by Rabinovitz and others (4-10). Striking similarities are that both kinds of inhibition are observed after prolonged incubation of the protein synthesizing system and in both cases, the inhibition can be overcome by

addition of excess initiation factor(s) (7). In the work reported in this paper, the incubation of the protein synthesizing system with f1 deacylates Met-$tRNA_f^{Met}$ and thus causes inhibition of protein synthesis initiation by limiting the concentration of charged Met-$tRNA_f^{Met}$. Upon addition of the peptide chain initiation factors, Met-$tRNA_f^{Met}$ forms the initiation complex with the initiation factor. The Met-$tRNA_f^{Met}$ in this complex is not degraded by f1 and can actively initiate protein synthesis.

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References

1. Woodley, C. L., Chen, Y. C., Bose, K. K., and Gupta, N. K., Biochem. Biophys. Res. Commun., 46, 839 (1972).
2. Chen, Y. C., Woodley, C. L., Bose, K. K., and Gupta, N. K., Biochem. Biophys. Res. Commun., 48, 1 (1972).
3. Woodley, C. L., Chen, Y. C., and Gupta, N. K., Methods in Enzymology, ed. by K. Moldave and L. Grossman, New York Academic Press (in press).
4. Maxwell, C. R., and Rabinovitz, M., Biochem. Biophys. Res. Commun., 35, 79 (1969).
5. Howard, G. A., Adamson, S. D., and Herbert, E., Biochim. Biophys. Acta, 213, 237 (1970).
6. Maxwell, C. R., Kamper, C. S., Rabinovitz, M., J. Mol. Biol., 58, 317 (1971).
7. Adamson, S. D., Yau, P. M., and Herbert, E., J. Mol. Biol., 63, 247 (1972).
8. Hunt, T., Vanderhoff, G. A., and London, I. M., J. Mol. Biol., 66, 471 (1972).
9. Gross, M., and Rabinovitz, M., Proc. Nat. Acad. Sci., U.S.A., 69, 1565 (1972).
10. Kaempfer, R., and Kaufman, J., Proc. Nat. Acad. Sci., U.S.A., 69, 3317 (1972).
11. Legon, S., Jackson, R. J., and Hunt, T., Nature, 241, 150 (1973).
12. Dettman, G. L., and Stanley, W. M., Jr., Biochim. Biophys. Acta, 287, 128 (1972).
13. Levin, D. H., Kyner, D., and Acs, G., Proc. Nat. Acad. Sci. U.S.A., 70, 41 (1973).

14. Morrisey, J., and Hardesty, B., Paper presented at the Protein Synthesis Meeting, Cold Spring Harbor Laboratory, September, 1972, abstract, p. 64.
15. Gupta, N. K., Chatterjee, N. K., Woodley, C. L., and Bose, K. K., J. Biol. Chem., 246, 7460 (1971).