

Equilibration of methionine into the Met-tRNA pool of rabbit reticulocyte lysates

Additional evidence that deacylation of ribosomal subunit-bound Met-tRNA_f does occur in heme-deficiency

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We have measured the rate of equilibration of [³⁵S]methionine into the Met-tRNA pool of rabbit reticulocyte lysate as in [FEBS Lett. (1982) 143, 301–305]. Our results indicate that hemin-deficiency inhibits the equilibration of methionine into the tRNA pool much less than protein synthesis or the equilibration of alanine into the tRNA pool, whereas cycloheximide inhibits these processes similarly. This finding is consistent with our previous data and supports the hypothesis that with hemin-deficiency much of the Met-tRNA_f that becomes bound to 40 S subunits subsequently undergoes enzymatic deacylation.

Reticulocyte lysate

*Hemin
Deacylation*

*Protein synthesis
Met-tRNA*

Translational control

1. INTRODUCTION

The translational control of protein synthesis by hemin in rabbit reticulocytes is mediated by the activation of a potent inhibitor of polypeptide chain initiation, termed the hemin-controlled translational repressor or HCR [1–4]. Polypeptide chain initiation is also suppressed by a low level of double-stranded RNA, which is due to the activation of a separate inhibitor termed dsI [5–8]. Both HCR and dsI act by phosphorylating the *M_r* 35 000 (α) subunit of eIF-2 [6–11], which results in a reduced, steady-state level of 40 S·Met-tRNA_f initiation complexes [4,6]. We have demonstrated that inhibition by HCR or dsI results in a marked increase in the steady-state level of 40 S·Met-tRNA_f·mRNA (48 S) complexes [12,13], suggesting that inhibition may also involve a reduced rate of combination of these 48 S complexes with

60 S subunits resulting, secondarily, in the deacylation of subunit-bound Met-tRNA_f [12] by a ribosomal Met-tRNA_f deacylase [14–16]. We have shown that the addition of highly purified Met-tRNA_f deacylase to lysate samples containing HCR or dsI reduces the [³⁵S]Met-tRNA_f labeling of 48 S complexes but has no effect on the level of [³⁵S]-Met-tRNA_f·40 S complexes occurring in the non-inhibited control [17].

One important piece of indirect evidence supporting deacylation that we have relied upon is our observation that under conditions where HCR or dsI reduce the rate of protein synthesis to about 5–10% of the control, the apparent rate of utilization of initiator tRNA for polypeptide chain initiation, as measured by the disappearance of exogenous [³⁵S]Met-tRNA_f or the incorporation into protein of formylmethionine from f[³⁵S]Met-tRNA_f, is only reduced to about 25–40% of the control [12,17]. Since 7-methyl-guanosine 5'-triphosphate and a low level of cycloheximide inhibit protein synthesis and the utilization of initiator

Abbreviations: HCR, hemin-controlled translational repressor; dsI, double-stranded RNA-activated inhibitor

tRNA to a similar degree [17], we interpreted these results to indicate that the rate of binding of Met-tRNA_f to 40 S subunits is reduced by HCR and dsI to only about 25–40% of the control and that as much as 80% of the Met-tRNA_f that becomes bound subsequently becomes deacylated, resulting in aborted initiation and a rate of protein synthesis that is only 5–10% of the control. However, in [18] the rate of equilibration of [³⁵S]methionine into the Met-tRNA pool was measured to estimate the rate of utilization of Met-tRNA_f for polypeptide chain initiation and data were obtained that appear inconsistent with our deacylation hypothesis [18]. We now report that, using the methodology in [18], we have obtained similar results but with a crucial and unexpected difference: the steady-state level of Met-tRNA_f in reticulocyte lysate is much lower in the presence of HCR than in its absence. When this is taken into account, the results obtained by this method agree with our previous findings and further support the idea that deacylation of subunit-bound Met-tRNA_f does occur with inhibition by HCR and dsI.

2. MATERIALS AND METHODS

Reticulocyte lysate was prepared from phenylhydrazine-treated rabbits as in [1,12]. Protein synthesizing samples contained 5 vol. lysate, 3 vol. master mix, and 3 vol. test solution or water. The master mix contained the following components in amounts to give the indicated concentrations in the final reaction mixture: KCl (75 mM), MgCl₂ (2 mM), ATP (0.5 mM), GTP (0.2 mM), creatine phosphate (15 mM), creatine phosphokinase (45 units/ml), leucine (0.3 mM) either as [¹⁴C]leucine (5 Ci/mol) or as [¹²C]leucine when a different radioactive amino acid was added, and the other 19 amino acids at concentrations corresponding to their relative abundance in rabbit globin. When added, hemin was at a concentration that was optimal for protein synthesis (about 25 μM). All incubations were at 34°C. The incorporation of a radiolabeled amino acid into protein was determined as in [12].

Partially purified HCR was prepared as in [17]. A separate protein activity termed the supernatant factor, which can completely reverse the inhibitory effect of hemin deficiency, was prepared from the post-ribosomal supernatant fraction [19]. EIF-2

was purified from reticulocyte ribosomes as in [20]. Reticulocyte tRNA was purified from the post-ribosomal supernatant fraction and deacylated as in [21].

The rate of equilibration of either [³⁵S]-methionine or of [³H]alanine into the lysate tRNA pool was determined essentially as in [18]. Following the addition of radioactive amino acid at 15 min of incubation, 25 or 30 μl aliquots of the reaction mixture were added directly to 150 μl water-saturated phenol and 125 μl 0.2 M sodium acetate (pH 5.2), 0.5% sodium dodecyl sulfate (at 0°C) and mixed thoroughly. After centrifugation, the epiphase was removed and the remainder was re-extracted with 75 μl 0.2 M sodium acetate (pH 5.2). The combined epiphases were mixed with about 0.1 mg rabbit reticulocyte, ribosomal RNA as carrier (to insure complete recovery of labeled tRNA), 75 μl 4 M NaCl, and 900 μl absolute alcohol and stored at –20°C overnight. The RNA was recovered by centrifugation, washed twice with 100 μl 95% alcohol, allowed to air dry, and then dissolved in 100 μl water. Aliquots (usually 50 μl) were then mixed with 0.50 ml 2 mg/ml, yeast RNA carrier and precipitated by mixing with 1.0 ml 2% (w/v) cetyltrimethylammonium bromide. These precipitates were collected by centrifugation, dissolved in 0.50 ml NCS solubilizer (Amersham), mixed with 10 ml scintillation fluid [21], and counted in a Tracor analytic, betatrac 6895 counter at an efficiency of 96% for ³⁵S and 62% for ³H.

All master mix reagents were from Sigma, [¹⁴C]leucine was from New England Nuclear, and [³⁵S]methionine and [³H]alanine were from Amersham.

3. RESULTS

The effect of hemin deficiency and of a limiting and saturating level of cycloheximide on protein synthesis in reticulocyte lysate at and after 15 min of incubation is shown in fig.1. Protein synthesis was inhibited to a similar degree (about 90%) by hemin deficiency or by the addition of HCR or of 0.45 μg/ml of cycloheximide to plus hemin samples, while inhibition was virtually complete with the addition of 125 μg cycloheximide/ml. From these results and from experiments where similar lysate samples were pulsed with [¹⁴C]leucine at the

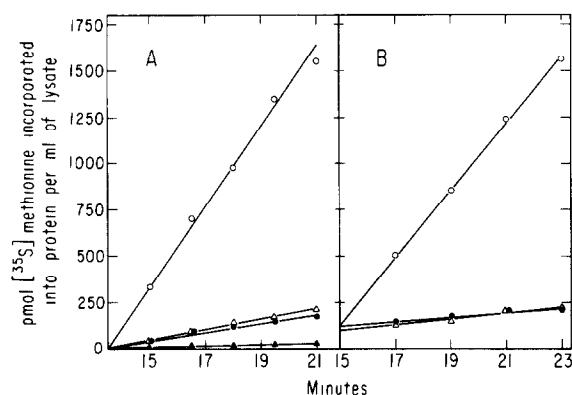


Fig.1. Rate of protein synthesis. Cell-free samples, containing 50 μ l lysate and 30 μ l master mix (with [12 C]leucine) in 110 μ l final vol., were incubated in the absence of hemin (\bullet), presence of hemin (\circ), or in the presence of hemin plus 0.45 μ g/ml (Δ) or 125 μ g/ml (\blacktriangle) of cycloheximide. After (A) 13.5 or (B) 15 min of incubation, 20 μ Ci [35 S]methionine were added to each sample (to give a final specific activity in the reaction mixture of 16×10^3 cpm/pmol), and 11 μ l aliquots were removed at the indicated times for the determination of acid precipitable radioactivity. The values in (A) have had the contribution from [35 S]Met-tRNA (determined separately under identical conditions; see fig.2) subtracted off, whereas the values in (B) represent the total, acid-precipitable radioactivity remaining after the aliquots were first treated with 0.1 M NaOH for 15 min to deacylate the tRNA. The small, zero-time background radioactivity in (B) may be secondary to the alkali treatment.

same time of incubation, we have determined that the rate of protein synthesis is reduced to 9%, 7% or 11% of that in the plus hemin control when incubation is in the absence of hemin, the presence of hemin plus a saturating level of HCR, or the presence of hemin plus 0.45 μ g cycloheximide/ml, respectively (table 1). We have examined whether under these conditions the addition of crude reticulocyte tRNA, at a level that we had used to measure the disappearance of [35 S]Met-tRNA_f or the incorporation into protein of formylmethionine from f[35 S]Met-tRNA_f [12,17], may itself influence the rate of protein synthesis by perturbing the lysate. We have found, however, that the addition of this level of tRNA, charged with cold methionine, has no effect on protein synthesis in the presence or absence of hemin (not shown).

Table 1

Effect of hemin deficiency, HCR, and cycloheximide on the relative rates of protein synthesis and equilibration of [35 S]methionine and [3 H]alanine into the tRNA pool

Conditions	Protein synthesis	[35 S]Met into Met-tRNA	[3 H]Ala into Ala-tRNA
+ Hemin	100	100	100
+ Hemin	8.9	36	10.2
+ Hemin + HCR	7.2	28	9.6
+ Hemin + 0.45 μ g/ml cycloheximide	11.1	9.3	7.6
+ Hemin + 125 μ g/ml cycloheximide	1.8	1.3	—

The relative rate of protein synthesis was determined from the data in fig.1 plus data from a separate experiment using [14 C]leucine. The relative rate of equilibration of [35 S]methionine and [3 H]alanine into tRNA was determined from the data in fig.2,4 and fig.3, respectively

The effect of hemin deficiency and cycloheximide on the rate of equilibration of [35 S]methionine into the Met-tRNA pool at and after 15 min of incubation is shown in fig.2. The results (left panel) indicate that equilibration in the presence of hemin was very rapid with a steady-state level of about 285 pmol Met-tRNA/ml lysate achieved within 2 min. The rate of equilibration, as judged by the initial time points, appears to be similar in samples incubated in the absence of hemin or in the presence of hemin plus 0.45 μ g cycloheximide/ml, which is comparable to the findings in [18]. However, the later time points demonstrate that while the sample with 0.45 μ g cycloheximide/ml reached an approximate steady-state level of 400 pmol Met-tRNA/ml of lysate after about 40 min, the hemin deficient sample achieved a steady-state level of only 175 pmol/ml in about 6 min. The result observed in the hemin-deficient sample is not a direct effect of hemin lack but is mediated by the activation of HCR, since a sample given hemin and HCR showed the same results as hemin deficiency, while another given a saturating level of the supernatant factor and no hemin behaved like the plus hemin sample (fig.4).

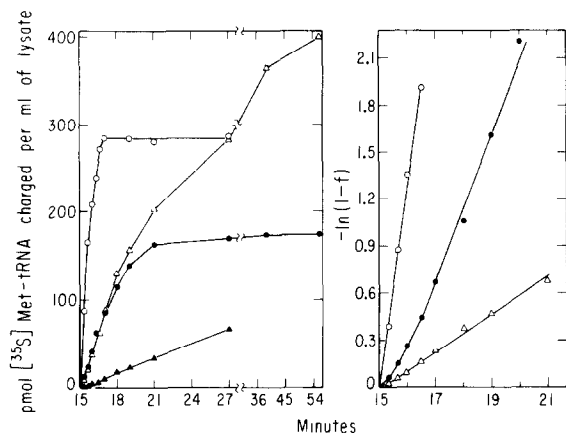


Fig.2. Rate of equilibration of [³⁵S]methionine into the Met-tRNA pool. Cell-free samples, containing 100 μ l lysate and 60 μ l master mix (with [¹²C]leucine) in 220 μ l final vol., were incubated in the absence of hemin (●), presence of hemin (○), or in the presence of hemin plus 0.45 μ g/ml (Δ) or 125 μ g/ml (\blacktriangle) of cycloheximide. After 15 min, 40 μ Ci [³⁵S]methionine were added to each sample (to give a final specific activity of 16×10^3 cpm/pmol), and 25 μ l aliquots were removed at the indicated times for the determination of [³⁵S]Met-tRNA as in section 2.

When equilibration is expressed as $-\ln(1-f)$ vs time (where f is the fraction of the steady-state level achieved at each indicated time; see fig.2, right panel) as in [18], the rate of equilibration of [³⁵S]methionine into the Met-tRNA pool is reduced to 9% of the plus hemin control in the presence of 0.45 μ g cycloheximide/ml, but to only 36% and 28% of the plus hemin control in the absence of hemin or presence of added HCR, respectively (table 1). These results indicate that the turnover of Met-tRNA, which is a measure of its utilization for protein synthesis, is reduced by HCR to only 28–36% of the control, while protein synthesis is reduced to 7–9% of the control (both processes are reduced to the same degree by cycloheximide). These findings are in excellent agreement with our measurements using [³⁵S]Met-tRNA_f [12,17] and provide additional support for our conclusion that as much as 75–80% of the Met-tRNA_f that becomes bound to 40 S subunits in the presence of HCR subsequently becomes deacylated by a ribosomal deacylase.

Although our measurement in fig.2 has been of

the total Met-tRNA and not Met-tRNA_f specifically, in [18] total Met-tRNA was found to be an approximate measure of Met-tRNA_f since this is the major tRNA^{met} present [18]. In addition, our results would indicate that the contribution of Met-tRNA_m should cause the observed difference between inhibition of protein synthesis and turnover of Met-tRNA in the presence of HCR to be slightly less than the actual difference between inhibition of protein synthesis and turnover of Met-tRNA_f. To obtain an estimate of the relative rate of equilibration of methionine into the Met-tRNA_m pool and to provide an additional control for the results in and conclusion from fig.2, we examined the rate of equilibration of [³H]alanine into Ala-tRNA, a representative elongator tRNA that is relatively abundant in rabbit reticulocytes [22]. The pmol [³H]Ala-tRNA charged/ml lysate is shown in the left panel, and the expression of the data as $-\ln(1-f)$ with time is shown in the right panel (fig.3). The results demonstrate that, as was the case with [³⁵S]methionine, the steady-state level of [³H]Ala-tRNA reached in the presence of 0.45 μ g cycloheximide/ml was somewhat higher than that in the plus hemin control, while that in the absence of hemin or in the presence of added HCR was much lower than the control level. The rate of equilibration of [³H]alanine into Ala-tRNA was very rapid in the plus hemin control and was reduced by cycloheximide to about 8% of the control (fig.3, right panel; table 1), similar to the inhibition by cycloheximide of protein synthesis and methionine equilibration into Met-tRNA. The rate of equilibration of [³H]alanine into Ala-tRNA in the absence of hemin or presence of hemin plus added HCR was reduced to about 10% of the plus hemin control, similar to the reduction in protein synthesis under these conditions but in sharp contrast to the effect on equilibration of methionine into Met-tRNA. Thus, the rate of equilibration or turnover of a representative elongator tRNA, which likely reflects the behavior of Met-tRNA_m as well, is inhibited by HCR to the same degree as protein synthesis and to a much greater degree than the equilibration or turnover of total Met-tRNA or, consequently, of Met-tRNA_f, further supporting the deacylation hypothesis.

The results in fig.2 and 3 have also suggested that HCR may reduce the formation of Met-tRNA and Ala-tRNA to a greater degree than their

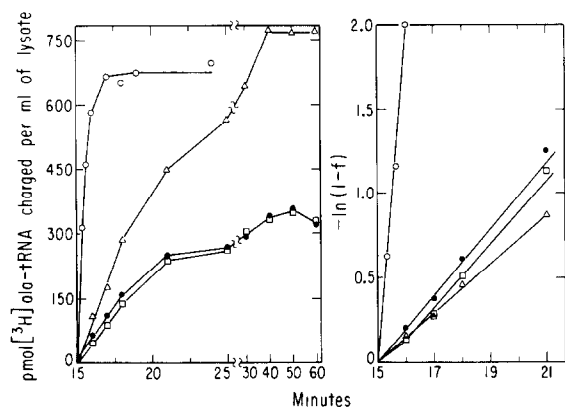


Fig.3. Rate of equilibration of [^3H]alanine into the Ala-tRNA pool. Cell-free samples, containing 120 μl lysate and 72 μl master mix in 264 μl final vol., were incubated in the absence of hemin (\bullet), presence of hemin (\circ), or the presence of hemin plus 0.45 μg cycloheximide/ml (triangles) or a saturating level of HCR (squares). After 15 min, 20 μCi [^3H]alanine were added to each sample (to give a final specific activity of 410 cpm/pmol), and 25 μl were removed at the indicated times to determine the level of [^3H]Ala-tRNA.

utilization for protein synthesis. Consequently, we attempted to measure the rate of formation of Met-tRNA in the lysate indirectly by adding a saturating level of cycloheximide, with or without additional tRNA, to samples which had been labeled with [^{35}S]methionine and had reached a steady-state level of [^{35}S]Met-tRNA. Since the cycloheximide should prevent any loss of Met-tRNA, the increase in [^{35}S]Met-tRNA should reflect the rate of charging of endogenous tRNA^{met} (no added tRNA), and that in samples receiving additional tRNA should reflect the rate of charging of the exogenous plus endogenous tRNA^{met}. The results (fig.4) from reactions given cycloheximide alone indicate that charging of the endogenous tRNA^{met} present in the sample with hemin was, after an initial lag, about 5.5-times faster than that in the sample without hemin. The actual difference in rate of charging between samples with and without hemin may be even greater than this, since the steady-state level of tRNA^{met} is greater in the absence than in the presence of hemin. The results from reactions given cycloheximide plus additional tRNA indicate that charging of exogenous tRNA^{met} was much more rapid than endogenous tRNA^{met} and that charging was, again, much faster

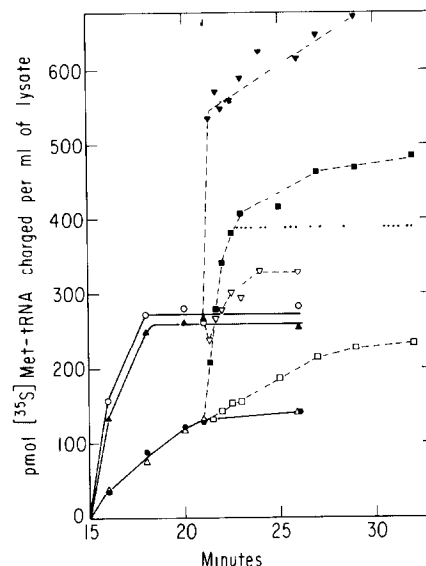


Fig.4. Use of saturating cycloheximide to estimate the rate of formation of [^{35}S]Met-tRNA. Cell-free samples, containing 120 μl lysate and 72 μl master mix in 264 μl final vol., were incubated in the presence of hemin (\circ), absence of hemin (\bullet), presence of hemin plus a saturating level of HCR (Δ), or in the absence of hemin plus a saturating level of the supernatant factor (\blacktriangle). After 15 min, 30 μCi [^{35}S]methionine were added to each sample (to give a final specific activity of 10×10^3 cpm/pmol), and 30 μl were removed at the indicated times to measure [^{35}S]Met-tRNA. Additional cell-free samples, prepared in an identical manner, were incubated in the presence (∇ , \triangledown) or absence (\blacksquare , \square) of hemin for 15 min and then given [^{35}S]methionine. At 21 min, all 4 samples received 125 μg cycloheximide/ml and 2 (∇ , \blacksquare) received 75 μg deacylated reticulocyte tRNA, and 30 μl were removed at the indicated times to determine the level of [^{35}S]Met-tRNA. The two dotted lines are an estimate of the formation of [^{35}S]Met-tRNA with the exogenous tRNA alone, obtained by subtracting the level seen with cycloheximide addition from that seen with the addition of cycloheximide plus tRNA.

in the presence than in the absence of hemin. These findings suggest that the steady-state level of Met-tRNA in the absence of hemin (or presence of HCR) is lower than in the presence of hemin because of a reduced rate of formation of Met-tRNA that is even greater than the reduction in its utilization.

4. DISCUSSION

Our findings have indicated that the rate of turn-

over of Met-tRNA_f, whether measured as the equilibration of [³⁵S]methionine into Met-tRNA as described in [18], or as the disappearance of exogenous [³⁵S]Met-tRNA_f [12,17], is reduced by hemin deficiency or added HCR to a much smaller degree than is protein synthesis (or turnover of an elongator tRNA), supporting the hypothesis that much of the Met-tRNA_f that becomes bound to 40 S subunits under these conditions subsequently undergoes enzymatic deacylation. We have also found, unexpectedly, that the steady-state level of Met-tRNA or of Ala-tRNA is much lower in the presence of HCR than in its absence despite the reduced rate of utilization of aminoacyl-tRNA and protein synthesis with HCR. In contrast, a level of cycloheximide, which inhibits protein synthesis to the same degree as HCR, produced, as would be expected, a greater steady-state level of Met-tRNA and Ala-tRNA than that seen in the non-inhibited control. The result with HCR appears due to a reduced rate of formation of Met-tRNA (to <20% of the control) that is greater than the reduction in its utilization (to about 30% of the control). The basis for this finding is at present unclear. We have observed no difference in the rate or extent of aminoacylation of reticulocyte tRNA with [³⁵S]methionine by reticulocyte charging enzymes, isolated as in [23], when either 25 μM hemin or highly purified HCR (at a level that would inhibit protein synthesis maximally in a similar volume of lysate) was added (not shown). This suggests that the effect of hemin deficiency (or HCR) on the formation of Met-tRNA is not a direct effect on the charging reaction but may be a secondary manifestation of HCR action in the lysate. In addition, the effect is not an irreversible loss of tRNA or enzyme, since the delayed addition of the supernatant factor or eIF-2 to samples without hemin, following the equilibration of [³⁵S]methionine into the Met-tRNA pool, resulted, in both cases, in an increase in the steady-state level of [³⁵S]Met-tRNA to a level that approached that of a sample with hemin (not shown). Although the basis for a reduced rate of aminoacylation of tRNA as a result of HCR action in the lysate is of interest and deserves further study, it cannot be a primary effect of HCR, since HCR produces a similar reduction in labeling of 40 S subunits with [³⁵S]Met-tRNA_f whether [³⁵S]methionine or [³⁵S]Met-tRNA_f is added.

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