

The turnover of methionine in the Met-tRNA pool and the control of protein synthesis in reticulocyte lysates

No evidence that haem-deficiency promotes deacylation of Met-tRNA_f

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1. INTRODUCTION

When rabbit reticulocyte lysates are incubated in the absence of haemin, the initial rate of protein synthesis is the same as in controls supplemented with haemin, but after ~5 min there is a severe and abrupt inhibition of initiation. Shortly before this shut-off there is a marked decrease in the level of the complexes between methionyl-tRNA_f and native 40 S ribosomal subunits that are thought to be an important intermediate in the pathway of initiation [1–4]. Since the absence of haemin does not affect the levels of methionyl-tRNA, GTP or native 40 S subunits in the lysate [1,5], the usual interpretation placed on these results is that the binding of methionyl-tRNA_f to 40 S subunits is inhibited. An alternative view which was first proposed in [6,7] is that the failure to detect 40 S • Met-tRNA_f complexes is largely due to a rapid deacylation of the Met-tRNA_f bound to the 40 S ribosomal subunits. This alternative model has since been refined in studies of lysates incubated without haemin or in the presence of the haem-controlled inhibitor (HCR), two situations which can be considered effectively the same [8]. Whilst the rate of protein synthesis was inhibited by 90–95% under these conditions, the rate of formation of 40 S • Met-tRNA_f complexes was only reduced by 70%, and when [³⁵S]methionyl-tRNA was added to the system the rate at which it disappeared was likewise inhibited by 70% if haemin was absent or HCR present [8]. From these observations the rate of binding of Met-tRNA_f to 40 S subunits was to be concluded only partially inhibited, and that 80% of the Met-tRNA_f bound to the subunits was rapidly

deacylated in a type of futile cycle which did not give rise to productive initiation events. Here, we use a more direct method to examine the rate of turnover of methionyl-tRNA in reticulocyte lysates incubated with HCR, and conclude that there is no significant futile cycle of deacylation. Our strategy could be more widely applied and used, for example, to measure the rate of translation in the presence of puromycin.

2. MATERIALS AND METHODS

[³⁵S]Methionine (1200 Ci/mmol) was from Amersham International, and emetine from Boehringer. The preparation of rabbit reticulocyte lysates, and of partially purified reticulocyte haem-controlled inhibitor (HCR) was as in [9]. Samples of lysates were preincubated at 30°C under protein synthesis conditions with 20 μM haemin present and in the presence or absence of emetine or HCR, but with radioactive amino acids omitted. After 10 min [³⁵S]methionine was added to give 0.2 mCi/ml final isotopic conc. and incubation continued. Samples (2 μl) were withdrawn at various times to determine incorporation into protein as in [5,9]. Samples (100 μl) were also taken into 1 ml 0.5% SDS 0.1 M sodium acetate–acetic acid buffer (pH 5.2) and extracted twice with an equal volume of water-saturated phenol. The RNA was precipitated from the aqueous phase with 70% ethanol, washed twice in 70% (v/v) ethanol 30% 0.2 M sodium acetate buffer (pH 5.2) and finally redissolved in water. Aliquots were taken for determination of the absorbance at 260 nm, and for precipitation

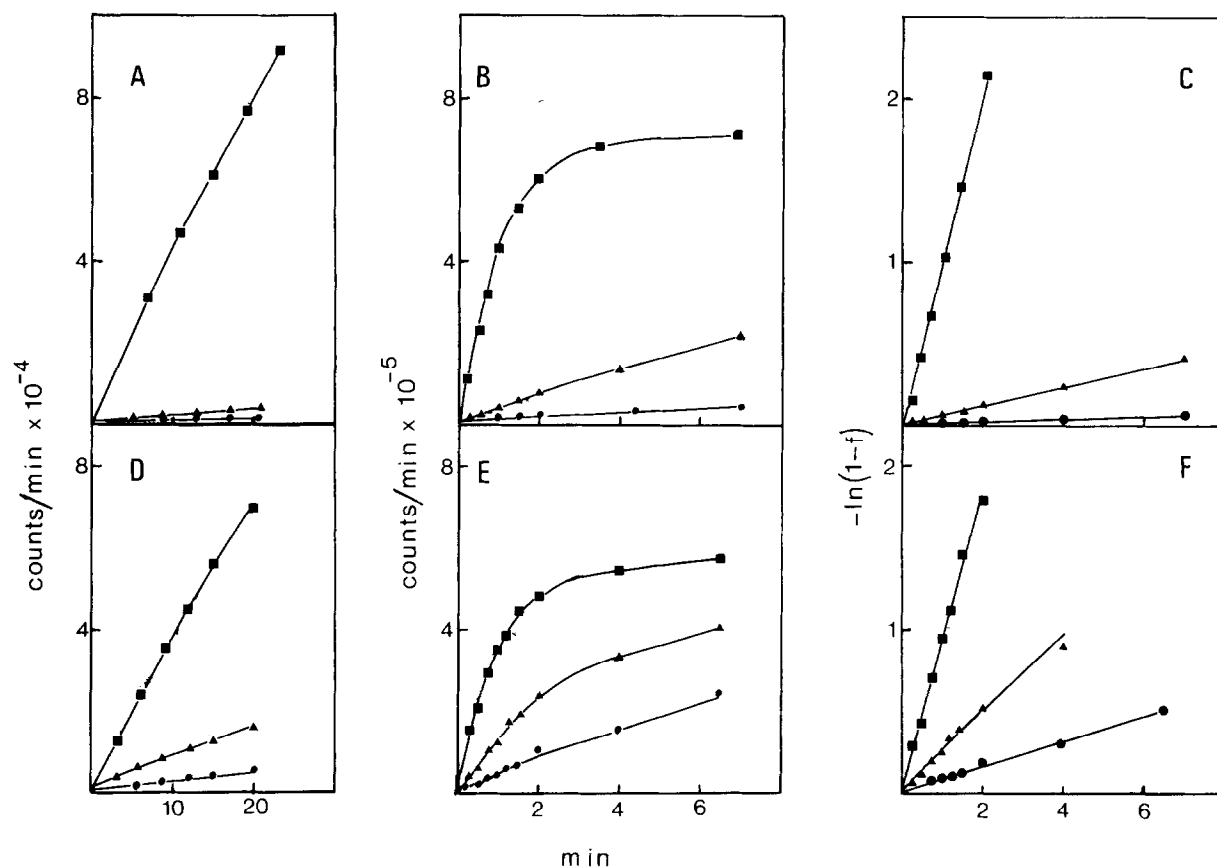


Fig. 1. Time-courses of [^{35}S]methionine incorporation into protein and into methionyl-tRNA: (A–C) HCR at 20 units/ml and emetine at 0.5 mM; (D–F) HCR at 2.5 units/ml and 1 μM emetine; (A,D) time-course of [^{35}S]methionine incorporation into protein expressed as cpm/ μl incubation mixture; (B,E) time-course of incorporation into methionyl-tRNA expressed as cpm/ A_{260} unit of RNA; (C,F) results of (B) and (E), respectively, replotted to show the rate of methionyl-tRNA charging as in the text. Assays containing HCR are shown by (\blacksquare); emetine by (\blacktriangle) and controls lacking inhibitors by (\bullet).

with cetyltrimethylammonium bromide for counting as in [2]. Samples of the RNA preparations were also digested with T1 ribonuclease (EC 3.1.27.3), and the ^{35}S -labelled fragments from Met-tRNA_m and Met-tRNA_f were separated by paper electrophoresis and counted, as in [1,2].

3. RESULTS

It should be possible to test the deacylation hypothesis by measuring the rate of entry of labelled methionine into the Met-tRNA pool in the lysate. The conversion of [^{35}S]methionine to [^{35}S]Met-tRNA depends on the availability of uncharged

tRNA^{Met}, which is governed by three different processes:

- (1) Utilisation of the Met-tRNA in protein synthesis;
- (2) Direct deacylation (either enzyme catalysed or not) of the charged tRNA;
- (3) Reversal of the aminoacylation reaction (though this would be highly disfavoured if inorganic pyrophosphate is kept at low levels by the action of pyrophosphatase).

In these experiments, the lysate was first preincubated with or without inhibitors under normal protein synthesis conditions, and labelled methionine added only when the system had achieved

Table 1
Relative rates of methionyl-tRNA charging and protein synthesis

| | Relative rate of: | |
|---------------------|-------------------------------|--------------------------------------|
| | [³⁵ S]Met incorp. | [³⁵ S]Met-tRNA labelling |
| Exp. 1 | | |
| HCR (20 units/ml) | 4.2% | 5.6% |
| Emetine (0.5 mM) | < 0.1% | 1.1% |
| Exp. 2 | | |
| HCR (2.5 units/ml) | 24.4% | 27.6% |
| Emetine (1 μ M) | 6.2% | 8.5% |

The rates of methionine incorporation into protein and of methionyl-tRNA charging in the presence of HCR or emetine are expressed as a percentage of the corresponding rates in control incubations lacking inhibitors: expt 1 refers to panels (A–C) of fig.1; expt 2 to panels (D–F).

steady state. Since lysates contain an appreciable pool ($\sim 5 \mu\text{M}$) of unlabelled methionine [10], essentially all the tRNA^{Met} should be present in the form of unlabelled Met-tRNA at the moment of addition of [³⁵S]methionine, and this addition should not significantly perturb the system since the increase in total methionine concentration is negligible.

Fig.1 shows the results of such an experiment. In controls where no inhibitor was present, entry of label into Met-tRNA was rapid, and complete by ~ 3 min, at which point the labelled methionine had equilibrated between the free methionine pool and the Met-tRNA pool. In contrast, when protein synthesis was totally inhibited by emetine, the initial rate of labelling of Met-tRNA was very much slower, and far from complete even at 10 min. To quantitate the rate of flux through the Met-tRNA pool, one can either estimate the initial slopes of the curves shown in fig.1 (B,E) or, as shown in fig.1 (C,F), replot the data as the function $-\ln(1-f)$ against time, where f is the fraction of the steady state labelling level, i.e., counts in Met-tRNA at time t /counts in Met-tRNA at time infinity. This gives a linear plot, whose slope is directly proportional to the flux. The rates determined by this approach are given in tabel 1 together with the rate of methionine incorporation into protein. There is an excellent correlation between the rate of protein synthesis and the rate of Met-tRNA charging. Thus,

when protein synthesis was inhibited $>99\%$ by high concentrations of emetine, the rate of charging is inhibited by 98%. It can be concluded that the high rate of Met-tRNA charging in controls lacking inhibitor is almost entirely dependent on the rate at which protein synthesis makes uncharged tRNA^{Met} available; only 2–3% of the tRNA^{Met} availability stems from deacylation of methionyl-tRNA or from reversal of aminoacylation, and $\geq 97\%$ originates from the utilisation of methionyl-tRNA in protein synthesis.

The important point is that when protein synthesis is inhibited by HCR the correlation between the rates of protein synthesis and the rate of charging is as good as when emetine is used as inhibitor (table 1). This rules out the possibility that HCR induces significant deacylation of Met-tRNA. To be specific, the model proposed in [8] would predict that when HCR inhibited protein synthesis by 95% (table 1, expt 1), the inhibition of Met-tRNA charging should be only ~ 65 –70%, which is clearly not the case.

It may be argued that the relevant issue is the rate of turnover of the initiator tRNA, Met-tRNA_f, and not the total Met-tRNA pool as was examined here. However, this is not a valid objection in this particular system since it has been reported that the tRNA^{Met} pool in reticulocytes is 85% tRNA_f^{Met} and 15% tRNA_m^{Met} [11,12], so that when large changes occur in the rate of charging of total methionyl-

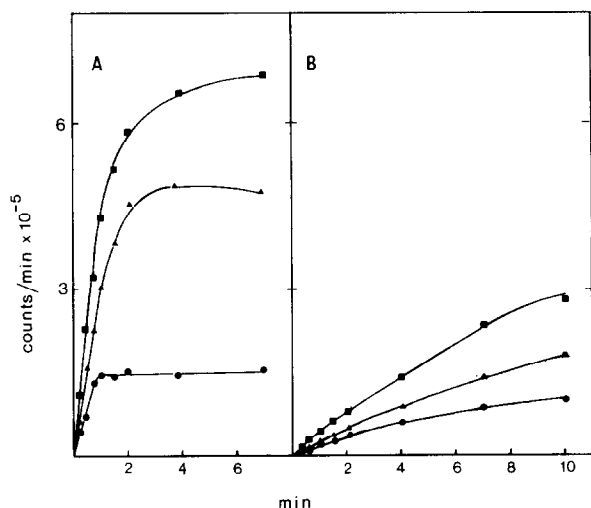


Fig.2. Time-course of $[^{35}\text{S}]$ methionine incorporation into Met-tRNA_f and Met-tRNA_m: incorporation into total Met-tRNA (●), into Met-tRNA_f (▲) and Met-tRNA_m (●) was assayed in the absence (A) or presence (B) of 20 units/ml HCR.

tRNA there must be correspondingly large changes in the rate of Met-tRNA_f^{Met} charging. We have analysed the rates of charging of the 2 individual species of tRNA^{Met} (fig.2), and although the additional manipulations involved introduce more scatter into the results, it is clear that the rate of charging of tRNA_f^{Met} is inhibited by HCR to about the same extent as the charging of total tRNA^{Met} (fig.2).

The relative levels of steady state labelling of the two species of Met-tRNA shown in fig.2 indicates that Met-tRNA_f represents ~80% of the total, slightly lower than the value obtained using a different method [11,12]. Since each globin chain has a single internal methionine residue [13] which originates from Met-tRNA_m, the synthesis of a globin chain uses equimolar amounts of the 2 species of methionyl-tRNA. The pool of Met-tRNA_m in reticulocyte lysates therefore turns over much faster than the 4-fold larger pool of Met-tRNA_f, and so the labelling of Met-tRNA_m reaches its steady state level more rapidly than is the case with Met-tRNA_f (fig.2).

4. DISCUSSION

There are 3 possible ways in which the absence of haemin or the presence of HCR could cause a decrease in 40 S • Met-tRNA_f complexes through a mechanism involving deacylation of the Met-tRNA in the complexes.

(1) The first molecule of Met-tRNA to bind to the 40 S subunits after onset of inhibition could be deacylated, the uncharged tRNA released from the subunit and no further methionyl-tRNA bound. This type of mechanism would be virtually impossible to detect, being essentially a single-cycle reaction which left no trace of itself on the 40 S subunit.

(2) A similar single-cycle process with the difference that the uncharged tRNA is retained on the 40 S subunit. As a test of this we have incubated lysates with purified $\text{t}^{[32\text{P}]\text{RNA}}_{\text{f}}^{\text{Met}}$ and then used sucrose gradient centrifugation to detect association of labelled tRNA with 40 S subunits. Although such association was seen in the case of lysates incubated with haemin, it was not found in lysates incubated without haemin or with double-stranded RNA [4]. We therefore believe that this model can be eliminated.

(3) That which is most consistent with [8]; a multi-cycle model in which methionyl-tRNA repeatedly binds to 40 S subunits and is then deacylated. This type of futile cycle has been eliminated by the experiments reported here. The reason for the discrepancy between our results and those in [8] is not clear, but we would argue that our methods involve less perturbation of the system than in [8] where $[^{35}\text{S}]\text{Met-tRNA}_f$ was added to the protein synthesis assays.

Therefore, we conclude that the decrease in 40 S • Met-tRNA_f complexes caused by haem-deficiency or the presence of HCR is the result of a decrease in the rate of formation of these complexes and not of an increase in the rate of abortive deacylation of Met-tRNA_f.

Our experiments show that the rate of protein synthesis can in effect be measured by determining the rate of charging of tRNA, and although this is a more indirect method than the usual approach there are situations where it is even indispensable.

For example, we have used it to estimate the rate of peptide bond synthesis in the presence of high concentrations of puromycin, a situation where amino acid incorporation into acid-precipitable protein cannot be used.

There is another general implication of these experiments which is of particular importance for the use of relatively inactive cell-free protein synthetic systems. If the pool of tRNA in such systems is already charged with unlabelled amino acids at the time when the radioactive amino acid is added at the start of the incubation it may take a long time before the labelling of the aminoacyl-tRNA reaches its steady state level, and until this steady state has been attained the observed rate of amino acid incorporation into protein will inevitably be an underestimate of the true rate of protein synthesis. When [^{35}S]methionine is used, it is the labelling of the Met-tRNA_m pool that is the important parameter, since methionine incorporation into acid-precipitable protein originates mainly from Met-tRNA_m with very little from Met-tRNA_f. In our experiments the labelling of the Met-tRNA_m pool in the absence of inhibitors reached a steady state level within 1 min (fig.2A). When high levels of HCR were added the labelling was much slower, but it had attained $\geq 70\%$ of the expected steady state level by 10 min (fig.2B), so that by measuring the incorporation rate over the time interval 10–20 min the degree to which the rate of protein synthesis has been underestimated in table 1 will be very small. It is only when 0.5 mM emetine was used that the errors would be serious, but since the rate of incorporation was essentially zero under these conditions (table 1), the true rate of protein synthesis can confidently be stated to be $< 1\%$ of the control rate. Therefore, these complications will not materially affect the conclusions that we have drawn from these experiments, but they serve to illustrate

the general point that the rate of amino acid incorporation is likely to underestimate the true rate of protein synthesis in situations where this rate is low.

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