INHIBITION OF AN INITIATION CODON FUNCTION BY HEMIN DEFICIENCY AND THE HEMIN-CONTROLLED TRANSLATIONAL REPRESSOR IN THE RETICULOCYTE CELL-FREE SYSTEM

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Received June 21, 1973

SUMMARY. The trinucleotide codon, AUG, promotes the combination of reticulocyte 40S and 60S native ribosomal subunits as well as recombination of those derived by dissociation factor. This interaction is inhibited by hemin deficiency or the hemin-controlled repressor and results in the loss of methionine from ribosomal bound met-tRNA$_f$. The locus of inhibition among the partial reactions of peptide chain initiation is between met-tRNA$_f$ and initiation codon binding to the 40S ribosomal subunit and peptide bond formation.

Globin synthesis in intact reticulocytes and their cell-free preparations is controlled by the availability of hemin (1-4). This regulation is mediated by a hemin-controlled repressor (HCR) of translation which is formed in extracts of reticulocytes incubated in the absence of hemin (5-7). The repressor is a protein of high molecular weight (8-11), and blocks the process of polypeptide chain initiation (10, 11).

Recent studies have indicated that the initiation step in mammalian protein synthesis involves the non-codon-directed binding of the initiator tRNA (met-tRNA$_f$) to the small ribosomal subunit (12-15). Such complexes of met-tRNA$_f$ and the 40S ribosomal subunit are found in a reticulocyte lysate protein-synthesizing system (12, 13, 16), and may be labelled by incubation of the system with radioactive methionine. The complexes can combine with the large ribosomal subunit in the presence of an exogenous mRNA (17) or the trinucleotide codon, AUG (18). This reaction, here termed the "shift reaction," may be assayed by the movement of methionine-associated radioactivity from the 40S to the 80S region of a sucrose density gradient. We wish to report that the AUG-promoted shift reaction is blocked by hemin deficiency and exogenous HCR,

and that the block is accompanied by deacylation of ribosome-bound initiator tRNA.

MATERIALS AND METHODS

Rabbit reticulocyte lysates were prepared as previously described (6). The protein-synthesizing system contained 5 volumes of a 1:1 water lysate, 3 volumes of master-mix, and 3 volumes of water or other additions. A concentration of hemin optimal for protein synthesis (25-35 μM) was included where indicated. The master-mix solution was prepared from ingredients stored at -20°C to yield the following final concentrations: KCl (75 mM), $Mg(CH_3COO)_2$ (2 mM), ATP (0.5 mM), GTP (0.2 mM), creatine phosphate (15 mM), creatine kinase (45 units/ml), and an amino acid mixture containing either 19 or 20 unlabelled amino acids. The composition of the amino acid mixture was the same as that reported for rabbit globin (19). Non-radioactive methionine was omitted from the amino acid mixture in experiments with [^{35}S]-methionine. The trinucleotide codon, AUG, and cycloheximide were added as indicated in individual experiments to promote subunit combination and inhibit endogenous protein synthesis, respectively.

Reaction mixtures were analyzed on 12 ml 15-30% (w/w) sucrose gradients containing buffer (10 mM Tris-HCl, pH 7.5; 30 mM KCl; 2 mM $Mg(CH_3COO)_2$. The gradients were centrifuged for 4-4.5 hr at 40,000 rpm (286,000 × g max.) in the Spinco SW41 rotor, and analyzed for radioactivity and absorption at 260 nm (16).

Crude HCR was prepared by incubation of reticulocyte post-ribosomal supernatant fraction in the absence of hemin (6). It was partially purified by CM-Sephadex and G200 Sephadex chromatography (9), and its inhibitory potency in units was estimated in a standard incorporating system (6).

Initiation factor activity was extracted from reticulocyte ribosomes in 0.55 M KCl and purified by DEAE-cellulose treatment. The material eluting between 0.1 M and 0.16 M KCl was collected (11). Protein and RNA concentrations were estimated as previously described (16).

[^{35}S]-L-methionine was purchased from the New England Nuclear Corporation, the trinucleotide codon, AUG, from Boehringer, and edeine from Calbiochem.

RESULTS

Addition of AUG to a lysate system containing hemin promoted the combination of the 40S and 60S ribosomal subunits. This lysate system was fortified for protein synthesis but was blocked in this process with cycloheximide to isolate the partial reaction. A marked shift of methionine-associated radioactivity from the 40S to the 80S region of the gradient was evident (Fig. 1A and 1B). In the presence of HCR, the

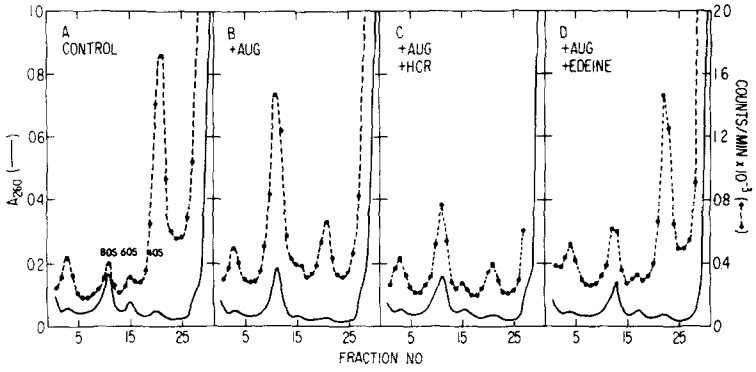

Figure 1. Effect of HCR and edeine on the AUG-promoted shift reaction in a lysate system. Aliquots (220 µl) of the protein-synthesizing system containing hemin (25 µM) and [^{35}S]-methionine (17.0 µCi/ml; 17.0 Ci/mmole) were incubated for 5 min at 34°C. Cycloheximide (final concentration 3.7 mM) was added and the incubation continued for a further 2 min. The following components were then added to give the indicated final concentrations and a volume of 250 µl: A) H_2O; B) AUG, 0.1 A_{260} unit/ml; C) AUG plus HCR, 200 units/ml; D) AUG plus edeine, 10^{-6} M. The reactions were terminated after a further 5 min by the addition of 400 µl ice-cold buffer and an aliquot (420 µl) was taken for sucrose gradient analysis.

combination of subunits was inhibited and the shift in radioactivity very much decreased (Fig. 1C). In addition there was a marked decrease in the total amount of radioactivity associated with the ribosome particles (40S plus 80S). Edeine (10^{-6} M) also blocked the reaction (Fig. 1D) but did not promote any loss of radioactivity from

the ribosomes. This suggests that edeine and HCR act at different loci.

A block of the shift reaction similar to that caused by HCR was evident in a protein-synthesizing system previously incubated in the absence of hemin. In such systems the concentration of 40S subunit/met-tRNA$_f$ initiation complexes is very low (13, 16). However, prevention of utilization of the complexes in protein synthesis with a high level of cycloheximide brings about considerable regeneration (16). Addition of AUG to such a system fails to promote a shift reaction, and the radioactivity associated with the 40S subunits almost completely disappears (Figs. 2A-2C).

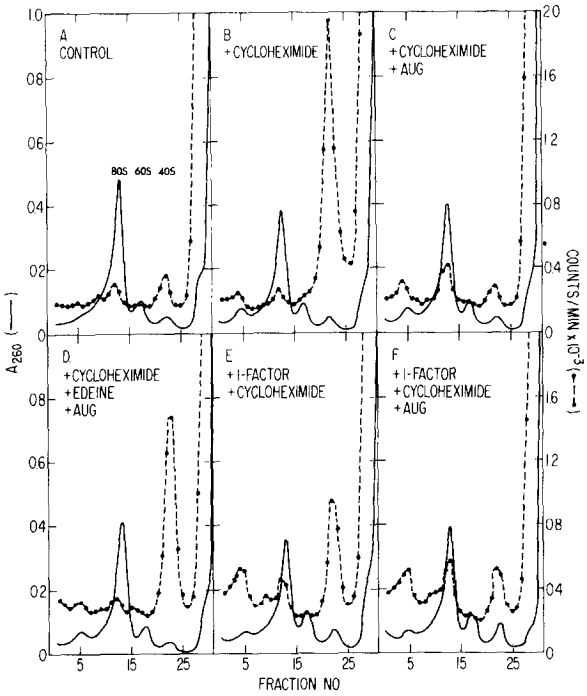

Figure 2. Effect of AUG in a lysate previously incubated in the absence of hemin. Aliquots (220 µl) of the protein-synthesizing system containing [^{35}S]-methionine (16.5 µCi/ml; 16.5 Ci/mmole) but no added hemin were incubated for 10 min at 34°C. The following components were then added to yield the indicated final concentrations and the incubation continued as described: A) H_2O, 13 min; B) cycloheximide, 3.3 mM, 13 min; C) cycloheximide, 10 min, then AUG, 0.37 A_{260} unit/ml, 3 min; D) cycloheximide, 8 min, then edeine, 2.7 × 10^{-6} M, 2 min, and then AUG, 3 min; E) I-Factor, 0.14 mg/ml, 10 min, then cycloheximide, 3 min; F) I-Factor, 10 min, then cycloheximide plus AUG, 3 min. The final volume of each mixture was 270 µl, and the reactions were terminated by the addition of 300 µl ice-cold buffer. An aliquot (400 µl) was taken for sucrose gradient analysis.

Edeine prevented the AUG dependent loss of ribosome associated radioactivity (Fig. 2D). Regeneration of the 40S initiation complexes was also achieved (Fig. 2E) in the presence of an initiation factor obtained by KCl extraction of reticulocyte ribosomes. This factor or factors (I-Factor) overcomes the effects of both hemin deficiency and HCR on protein synthesis (10, 11), exhibits met-$tRNA_f$ binding (16) and ribosome-dissociation (20) activities, but it did not promote the shift reaction (Fig. 2F).

In many lysate preparations the concentration of native ribosomal subunits was too low to permit estimation of the absorbancy changes associated with the shift reaction. This difficulty could be surmounted by including the ribosome-dissociation factor (20) in the incubations. With this modification the optical density changes seen in the presence of AUG were very pronounced (Fig. 3A and 3B). The effect of HCR could be demonstrated even when it is added after the shift had occurred (Fig. 3B-3E). This indicates either a direct role of HCR in the breakdown of the 80S initiation complex, or alternatively, an inhibition of the forward reaction followed by a displacement of equilibrium.

In addition to inhibition of the shift reaction itself (Figs. 1D and 3F), edeine also prevented the loss of 40S subunit-associated radioactivity observed as a result of HCR inhibition (Fig. 4). A similar protective effect of edeine was seen in the hemin-deficient system (Fig. 2D).

The loss of methionine from the small ribosomal subunit might represent either complete release of bound met-$tRNA_f$ or its deacylation. This was investigated by estimating the total amount of $tRNA_f^{Met}$ associated with the small ribosomal subunits of control and inhibited protein-synthesizing systems. The amounts of $tRNA_f^{Met}$ present were very similar in all cases (Table I), and the values obtained were comparable to previous estimates (12, 16).

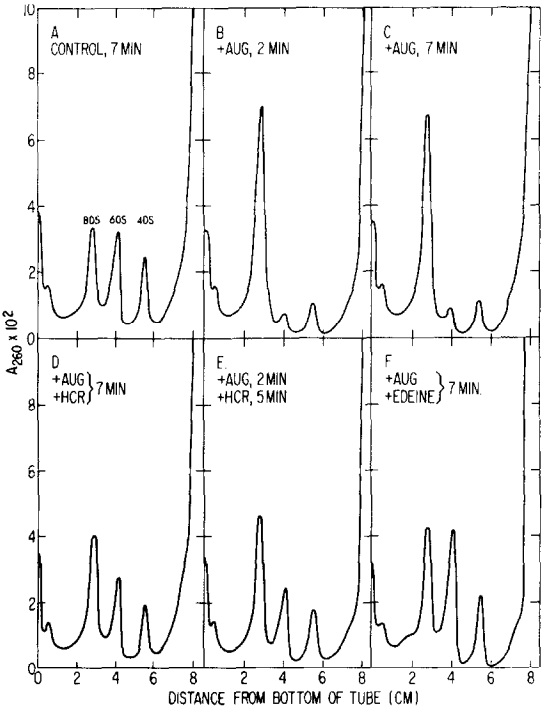

Figure 3. The effect of HCR on the AUG-promoted shift reaction of ribosomal subunits formed by the dissociative activity of I-Factor. Aliquots (110 μl) of the protein-synthesizing system containing hemin (35 μM) and I-Factor (0.16 mg/ml) were incubated for 5 min at 34°C. All samples then received cycloheximide (final concentration 3.3 mM) and the following additional components in a final volume of 130 μl: A) H_2O for 7 min; B) AUG, 0.37 A_{260} unit/ml, for 2 min; C) AUG for 7 min; D) AUG plus HCR, 162 units/ml, for 7 min; E) AUG for 2 min, then HCR for 5 min; F) AUG plus edeine, 2.7×10^{-6} M, for 7 min. The reactions were terminated by the addition of 250 μl ice-cold buffer, and an aliquot (190 μl) taken for sucrose gradient analysis.

DISCUSSION

The combination of the native ribosomal subunits in the presence of the trinucleotide AUG is a model reaction for an intermediate stage in peptide chain initiation, since it can occur in the presence of cycloheximide, which inhibits synthesis of the first peptide bond (21). The data suggest that this partial reaction is the primary site of action of the hemin-controlled repressor. The fact that a similar block is evident in a hemin-deficient system lends support to this view.

Previous studies employing labelled methionine in a complete protein-synthesizing system demonstrated that HCR caused a disappearance of met-$tRNA_f$/40S

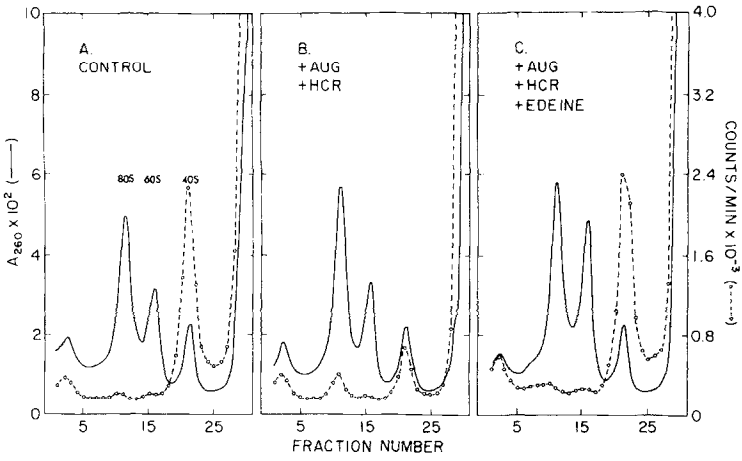

Figure 4. The effect of HCR and edeine in combination. Aliquots (110 µl) of the protein-synthesizing system containing hemin (35 µM), I-Factor (0.16 mg/ml) and [^{35}S]-methionine (36.0 µCi/ml; 110 Ci/mmole) were incubated for 5 min at 34°C. The samples then received cycloheximide (final concentration 3.3 mM) and the following additional components in a final volume of 135 µl: A) H_2O; B) AUG, 0.37 A_{260} unit/ml, plus HCR, 310 units/ml; C) AUG plus HCR plus edeine, 2.7×10^{-6} M. The mixtures were incubated for an additional 5 min at 34°C and the reactions terminated by the addition of 250 µl ice-cold buffer. An aliquot (190 µl) was taken for sucrose gradient analysis.

TABLE I

Effect of Hemin Deficiency and HCR on the Level of $tRNA_f^{Met}$ on 40S Ribosomal Subunits

The protein-synthesizing system (12.1 ml) was incubated for 15 min at 34°C either A) with hemin, 35 µM, B) with hemin and HCR, 62 units/ml, or C) without hemin. Native 40S ribosomal subunits were separated by zonal centrifugation and RNA extracted from them with phenol and SDS (16). The deacylated RNA was incubated under the conditions previously described (16) with [^{35}S]-methionine (0.48 µCi/ml; 240 Ci/mole) and E. coli Q13 aminoacyl-tRNA synthetases, which charge rabbit $tRNA_f^{Met}$ but not $tRNA_M^{Met}$. A blank value (no added RNA) of 3.0 has been subtracted from experimental values.

Incubation condition	Acceptor capacity (p moles methionine/mg RNA)
With hemin	282
With hemin and HCR	307
Without hemin	269

subunit initiation complexes (13, 16). Legon et al. (13) interpreted this to be due to an inhibition of binding of met-$tRNA_f$ to the small ribosomal subunit. However, HCR did not affect binding of met-$tRNA_f$ to 40S subunits in an initiation factor-catalyzed partial reaction containing cycloheximide (16). Moreover, direct analysis of native 40S subunits of control and inhibited systems revealed no difference in the level of $tRNA_f^{Met}$ (Table I). A hypothesis consistent with these observations and the results reported here is that HCR inhibition of peptide chain initiation involves deacylation of the met-$tRNA_f$ already bound to the small ribosomal subunit. In the presence of cycloheximide, this HCR-promoted deacylation is completely dependent upon added AUG. The stabilizing effect of cycloheximide on the initiation complexes thus appears not only to be due to its role in preventing their utilization for protein synthesis, but also to the masking of the initiation codon of natural mRNA by ribosomes blocked in translation. The protective effect of edeine (Fig. 4) is consistent with the observation that the antibiotic inhibits binding of AUG to 40S subunits (22).

Morrisey and Hardesty (23) have described an enzyme, met-$tRNA_f$ hydrolase, which deacylates the initiator tRNA in a reticulocyte cell-free system. The reaction required the presence of both AUG and 40S subunits, and was strongly inhibited by the addition of 60S subunits. In view of its substrate specificity, the enzyme is a likely candidate to be involved in HCR inhibition. This enzyme may be the same as that recently reported by Gupta and Aerni (24). It is possible that HCR stimulates the activity of the enzyme on the 40S subunit. Alternatively, deacylation in the inhibited system may simply be a consequence of the failure of the 40S subunit to combine with the 60S subunit. Further investigation will be required to distinguish between these models.

This investigation was conducted during the tenure of a Damon Runyon Cancer Research Fellowship to K. B. The present address of S. M. is Department of Antibiotics, National Institute of Health of Japan, 10-35, Kamiosaki 2 chome, Shinagawa-ku, Tokyo 141, Japan.

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