Inhibition of Synthetic and Natural Messenger Translation

I. PURIFICATION AND PROPERTIES OF A PROTEIN ISOLATED FROM ESCHERICHIA COLI MRE 600 RIBOSOMES*

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SUMMARY

An acidic protein isolated from the 1.0 M NH₄Cl wash of Escherichia coli MRE 600 ribosomes was found to inhibit translation of R17 RNA or poly(U) and initiation complex formation with R17 RNA. The effect of this protein on poly(U) translation was used as the basis of a simple and specific assay for its purification. The factor was purified to homogeneity by chromatography on DEAE-cellulose, hydroxypatite, and Sephadex G-150, and its molecular weight was determined to be 69,000. Factor activity was rapidly lost above 50°, and was not inactivated by treatment with p-hydroxymercuribenzoate. An affinity of the purified protein for RNA was shown by the formation of an RNA-protein complex which could be retained on Millipore filters. On the basis of molecular weight, mobility on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and inhibitory activity in R17 RNA and poly(U) translation, the factor was identified as subunit I of Q₃ replicase.

Initiation of protein synthesis in extracts of Escherichia coli requires the addition of three protein factors, IF-1, IF-2, and IF-3, which have been purified to homogeneity and extensively studied in recent years (1–3). All three factors are extracted from the 1.0 M NH₄Cl wash of E. coli ribosomes. During the purification of IF-2, crude fractions were found to inhibit poly(U) and R17 RNA translation. An acidic protein was isolated from the 1.0 M NH₄Cl ribosomal wash, and on the basis of molecular weight, mobility on SDS-gel electrophoresis, and inhibitory activity in R17 RNA and poly(U) translation, the factor was identified as subunit I of Q₃ replicase (4). This communication describes a simple and specific assay for this protein, its purification to homogeneity, and some of its properties. Throughout this report, the factor is referred to as subunit I. A similar protein which inhibits natural messenger translation was previously isolated (5–7).

MATERIALS AND METHODS

Hydroxypatite (Bio-Gel HTP) was purchased from Bio-Rad Laboratories, and H-labeled poly(U) and poly(A) were obtained from Miles Laboratories. All other chemicals were purchased from standard sources (7, 8). Pure Q₃ replicase was from T. Blumenthal and K. Weber of Harvard University, and 3P-labeled R17 RNA was from J. A. Steitz of Yale University. Frozen E. coli MRE 600 cells, grown to late log phase, were labeled in the methionine, and R17 RNA was prepared as previously described (8, 9). Protein was determined by the method of Lowry et al. (10).

Ribosomes IF-1, IF-2, and IF-3—Ribosomes were prepared from freshly grown E. coli MRE 600 cells and were washed with 1.0 M NH₄Cl (8). IF-1 and IF-2 used in R17 RNA translation were isolated from the R17 RNA (8, 9). IF-1 and IF-2 were utilized in initiation complex formation with R17 RNA (8, 9). IF-3 was purified as in previous work (9).

Assay of Subunit I by Inhibition of Poly(U)-dependent Phenylalanine Incorporation—The inhibitory effect of subunit I on poly(U)-dependent polyphenylalanine synthesis provided a simple and rapid assay for this protein during purification. Each assay contained, in a volume of 0.125 ml, 100 mM NH₄Cl, 15 mM magnesium acetate; 16 mM 2-mercaptoethanol; 63 mM Tris-HCl, pH 7.2; 0.3 mg of E. coli MRE 600 S-15 dialyzed fraction; 1.3 mM ATP; 0.3 mM GTP; 17 mM phosphocreatine; 8 μg of creatine kinase; 0.1 μM [U-14C]phenylalanine (10 μCi per mmole); 4 μg of poly(U); 5 A₂₆₀ units of E. coli MRE 600 ribosomes; 50 μg of E. coli W tRNA; and 2.5 to 5.0 μg of Stop 5 IF-3 (9). The reaction was initiated by the addition of poly(U). The amount of inhibition by subunit I did not vary if the order of addition of reactants was altered. After incubation for 20 min at 37°, the reaction was terminated by the addition of 5% trichloroacetic acid. The samples were then heated for 15 min at 90°, the precipitate was collected on Millipore filters, washed with 3 ml of 5% trichloroacetic acid, dried, and radioactivity measured in a Packard Tri-Carb liquid scintillation spectrometer with Liquifluor as solvent. One unit of activity was defined as 10% inhibition of the standard assay of poly(U)-dependent phenylalanine incorporation. Specific activity of subunit I is expressed as units per mg of protein. At very low concentrations of subunit I, inhibition was not a linear function of protein added (Fig. 1). This effect may be due to the requirement for several molecules of subunit I per ribosome to inhibit the reaction. Accordingly, the amount of protein which gave 20% inhibition was routinely employed to calculate the specific activity and yield. Recently, the amount of inhibition by subunit I was found to increase when ribosomes used in the assay were washed twice in 1.0 M NH₄Cl for a total period of 16 hours. This increase in inhibitory activity is presently under investigation. Possibly fragments of mRNA or tRNA are removed from the ribosomes by longer exposure to 1.0 M NH₄Cl. Subunit I was also found to be

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The abbreviations used are: SDS, sodium dodecyl sulfate; p-HMB, p-hydroxymercuribenzoate.
active at all temperatures where poly(U) translation could be detected.

Initiation Complex Formation and Translation of R17 RNA—Formation of a 70S initiation complex with R17 RNA was detected by sucrose density centrifugation (8), and translation of R17 RNA was assayed as previously described (9).

Binding of Labeled RNA to Protein—Formation of a complex between subunits 1 or IF-3 and labeled RNA was measured by the retention of the radioactive complex on a Millipore filter. Each assay contained, in a volume of 50 µl, 100 mM NH₄Cl; 20 mM Tris-HCl, pH 7.6; 10 mM magnesium acetate; 1.0 mM dithiothreitol; 1 A₃₆₀ unit of 32P-labeled R17 RNA; 50 µg of bovine serum albumin; and 100 µg of crude IF-3 or IF-2 (II), or 100 µg of Step 5 IF-3 (9). The discovery that the same fractions also inhibited R17 RNA translation (Table I). This inhibitory activity was subsequently localized in the 35 to 45% (NH₄)₂SO₄ fraction; 0.3 µg of IF-2; 7.3 µg of IF-2 (11); 1.8 µg of Step 5 IF-3 (9). The blank in the absence of factors, 22 pmoles, was subtracted from each value.

Inhibition of R17 RNA translation by different fractions derived from the ribosomal wash

The conditions of the R17 RNA-dependent assay are as described under "Materials and Methods." Each reaction mixture contained 5 A₉₀₀ units of ribosomes; 1 A₉₀₀ unit of R17 RNA; 0.3 µg of IF-1; 7.3 µg of IF-2 (11); 1.8 µg of Step 5 IF-3 (9). The inhibition of translation was assayed as described under "Materials and Methods." Each reaction mixture contained, in a volume of 50 µl, 100 mM NH₄Cl; 20 mM Tris-HCl, pH 7.6; 10 mM magnesium acetate; 10 mM 2-mercaptoethanol (Buffer A). The cell suspension was disrupted by three consecutive passages through a Manton-Gaulin homogenizer. Debris was removed by centrifugation for 60 min at 12,000 rpm in the Sorvall GSA rotor. The clear supernatant (S30 extract) was stored frozen in 500-ml aliquots at −80°C. The S30 extract was thawed in 500-ml batches, and treated with DNaS (3 µg per ml) for 10 min at 0°C. The solution was then clarified by centrifugation for 60 min at 12,000 rpm in the Sorvall GSA rotor. Ribosomes were prepared by centrifugation of the extract for 2 hours at 60,000 rpm in the Spino 60 Ti rotor. The ribosomal pellet was stirred overnight in 1 liter of buffer containing 1.0 M NH₄Cl; 20 mM Tris-HCl, pH 7.8; 10 mM magnesium acetate; 10 mM 2-mercaptoethanol (Buffer B). The ribosomes were collected by centrifugation in the 60 Ti rotor as described above, and the supernatant (1.0 M NH₄Cl wash) used as the source of subunit I. 

Ammonium Sulfate Fractionation—The 1.0 M NH₄Cl wash was precipitated with (NH₄)₂SO₄ to 35% saturation (10.4 g/100 ml of solution), stirred for 20 min, and centrifuged in the GSA rotor at 12,000 rpm for 20 min. The supernatant was discarded and the supernatant brought to 45% saturation with ammonium sulfate (5.7 g/100 ml) and stirred for 20 min. The precipitate was collected by centrifugation, and used as a source of IF-2. The supernatant was brought to 55% saturation with ammonium sulfate (5.9 g/100 ml), stirred for 20 min, and the precipitate collected as described above. This fraction (45 to 55%) possessed most of the inhibitory activity, and did not contain significant amounts of IF-2 and IF-3. The precipitate was dialyzed overnight against buffer containing 20 mM NH₄Cl; 20 mM Tris-HCl, pH 7.8; and 0.2 mM magnesium acetate (Buffer C). Any insoluble material was removed by centrifugation for 20 min at 16,000 rpm, and the supernatant was stored at −80°C.

DEAE-cellulose Chromatography—The 45 to 55% (NH₄)₂SO₄ fraction from 1.5 kg of cells was applied to a column (2.5 × 100 cm) of DEAE-cellulose, previously equilibrated with Buffer C.
The column was first washed with 1 liter of Buffer C at a flow rate of 1 ml per min, then a linear gradient of 70 mM to 350 mM NH₄Cl was applied in a total volume of 2 liters of Buffer C. Fractions containing 10 ml were collected. Inhibitory activity was eluted from the column at 0.23 M NH₄Cl.

Hydroxypatite Chromatography. The fractions from the DEAE-cellulose column containing activity were pooled, dialyzed overnight against buffer containing 10 mM potassium phosphate, pH 7.5 (Buffer D), and applied to a column of hydroxypatite (1.5 x 30 cm) previously equilibrated with Buffer D. After washing the column with 3 volumes of Buffer D, at a flow rate of 0.25 ml per min, a linear gradient of 10 to 250 mM potassium phosphate was applied in a total volume of 350 ml of Buffer D. Inhibitory activity eluted from the column at 70 mM potassium phosphate.

Sephadex G-150 Chromatography—The fractions from the hydroxypatite column containing inhibitory activity were pooled, dialyzed overnight against Buffer C, and applied to a column of Sephadex G-150 (1.5 x 90 cm) equilibrated with Buffer C. The column was washed with Buffer C at a flow rate of 0.068 ml per min until all included protein was eluted. Fractions containing inhibitory activity were pooled and stored at 5°C (Fig. 2).

Comments on Purification and Properties of Subunit I—A summary of the purification procedure is given in Table II. At Step 4, essentially homogeneous subunit I was recovered in 24.5% yield with a specific activity of 431. The increase in activity during purification is shown in Fig. 1. Subunit I could not be assayed in the 1.0 M NH₄Cl ribosomal wash. Even at Step 1, an inhibitory effect was observed only when large amounts of protein were added.

Subunit I was found to be stable for 4 weeks at 5°C when stored in Buffer C. Fractions could be frozen at -80°C and thawed with less than 10% loss of activity. A very low level of RNase II (14) was observed in Step 4 preparations of subunit I. Under the conditions of the nuclease assay, 0.09 μg of poly(A) or 0.21 μg of poly(U) was degraded in 60 min by 1 μg of Step 4 subunit I. Similar low activities of RNase II were also observed in preparations of Step 5 IF-3 (8) and Qβ replicase (16). No degradation of R17 RNA was observed when Step 4 subunit I was incubated with the RNA for 15 min at 37°C, and the mixture was analyzed on a linear 5 to 20% sucrose gradient.

Step 4 subunit I has an A₂₆₀/A₂₈₀ ratio of 1.59 and was found to be 93.3% pure as determined by densitometer tracing of SDS-polyacrylamide gels. The pure protein migrated on SDS-polyacrylamide gels with the same mobility as subunit I of Qβ replicase (Fig. 3). Subunit I could be identified as a prominent band on gel electrophoresis of the proteins in the 1.0 M NH₄Cl ribosomal wash, and represents approximately 5.4% of the total protein removed from the ribosomes by this procedure. The molecular weight of subunit I is 69,000 as determined by SDS-polyacrylamide gel electrophoresis according to the procedure of Weber and Osborn (13).

Thermal Stability of Subunit I—To determine the stability of subunit I to heating, samples of the protein were incubated in Buffer C for 5 min at different temperatures. Subunit I was stable up to 50°C and activity was rapidly lost above this temperature.

Sensitivity of Subunit I to p-HMB—Samples of Step 4 subunit I were incubated at 25°C for 30 min with 0.1 mM p-HMB, dialyzed overnight against Buffer C, and assayed for activity in the poly(U) translation system. No reduction in activity was observed after incubation with p-HMB. This result indicates that free sulfhydryl groups are not required for the activity of subunit I in poly(U) translation.

Inhibition of R17 RNA Translation by Subunit I—At all steps of purification, subunit I could be detected by its effect on poly(U) or R17 RNA translation. Greater than 85% inhibition of R17 RNA translation (Fig. 4) was observed with saturating levels of Step 3 subunit I (above 45 μg per assay).

Inhibition of Initiation Complex Formation by Subunit I—The inhibitory effect of subunit I on the formation of an initiation complex containing phage RNA, 30 S and 50 S ribosomal subunits and fMet-tRNA₁ (1, 2). Since subunit I inhibits the translation process, it was important to determine whether this protein also had an effect on initiation complex formation. The results shown in Table III demonstrate that inhibition of R17 RNA-dependent ribosomal binding of [³⁵S]Met-tRNA occurs in the presence of subunit I, and increases linearly with increasing levels of protein.

Binding of R17 RNA to Subunit I—Another property of subunit I, which may be of importance in its mechanism of action, is the ability to bind RNA. When purified subunit I was incubated with R17 [³⁵S]RNA, as described in Fig. 5, radioactivity was retained on Millipore filters. A similar property was previously reported for IF-3 (1). In this experiment, several molecules of subunit I were required to bind 1 molecule of R17 RNA. The exact number of binding sites on the RNA molecule should not be inferred from these data, as partial autolytic cleav-

![Fig. 2. Chromatography of subunit I (Step 3) on Sephadex G-150. Aliquots of each fraction (30 μl) were assayed for inhibitory activity in the poly(U) translation system as described under "Materials and Methods."](image-url)
FIG. 4. Inhibition of R17 RNA translation by subunit I. The inhibitory activity of Step 3 subunit I in R17 RNA translation was assayed as described under "Materials and Methods."

TABLE III

Inhibition of R17 RNA-dependent ribosomal binding of fMet-tRNA by subunit I

The procedure for determination of initiation complex formation with R17 RNA is described under "Materials and Methods." Each reaction mixture contained 8.5 μg of IF-1; 0.3 μg of IF-2-ω (8); 5.9 μg of Step 5 IF-3 (9); 35 pmoles of [14C]fMet-tRNA; 2 A260 units of R17 RNA; 5 A260 units of ribosomes; and subunit I (Step 2 fraction) as indicated. After incubation for 15 min at 37°C, each assay was analysed for 70 S initiation complex formation by sucrose density gradient centrifugation as previously reported (8). The blank in the absence of factors, 0.55 pmole, was subtracted from each value.

<table>
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DISCUSSION

The 1.0 M NH₄Cl wash of E. coli ribosomes contains three protein factors, IF-1, IF-2, and IF-3, which are required for polypeptide chain initiation. A multitude of other proteins, some of which are involved in protein synthesis, appear to be extracted from the ribosomes with high salt. RNase I and II (14, 17) and EF-G (18) are among the nonstructural proteins which have been found on the surface of the ribosomes. Exposure of the ribosomes to 1.0 M NH₄Cl has also led to the release of certain structural proteins (19).

* M. Miller and A. J. Wahba, unpublished results.

Fig. 3. SDS-polyacrylamide gel electrophoresis of subunit I and Q8 replicase. Gel electrophoresis was carried out according to the procedure of Weber and Osborn (13). A, 3.6 μg of Q8 replicase; B, 1.4 μg of Step 4 subunit I.

Fig. 5. Binding of R17 [32P]RNA by subunit I and IF-3. The binding of [32P]labeled R17 RNA to protein was measured by retention of the RNA-protein complex on Millipore filters as described under "Materials and Methods." ○, R17 [32P]RNA bound to membrane in the presence of increasing levels of Step 4 subunit I. ●, R17 [32P]RNA bound to membrane in the presence of increasing levels of Step 5 IF-3 (9). A blank in the absence of protein, 0.12 pmole, was subtracted from each value.
The inhibitory activity for R17 RNA translation, initially detected in crude fractions of IF-2, appeared to be due to contamination by another protein. Highly purified IF-2α and IF-2β (8), when added in saturating amounts, did not inhibit R17 RNA translation. A protein factor, with inhibitory properties in R17 RNA and poly(U) translation, was isolated from the 1.0 M NH₄Cl wash of E. coli MRE 600 ribosomes. At all stages of purification, factor activity could be detected by inhibition of amino acid incorporation with either poly(U) or R17 RNA. This protein was also found to suppress initiation complex formation with R17 RNA. Inhibition was not due to contamination with RNase I or II, for neither activity was detected in significant amounts in highly purified preparations. The factor was found to be unstable above 50°C, and was not inactivated by preincubation with p-HMβ.

We have referred to this factor as subunit I for several reasons. Its mobility in SDS-gel electrophoresis and its molecular weight are identical with that of subunit I of Qβ replicase. In addition, we observed that addition of 7 μg of pure Qβ replicase resulted in 35% inhibition of poly(U)-dependent polyphenylalanine synthesis, and 18% inhibition of R17 RNA-directed lysine incorporation. The inhibition by Qβ replicase detected in these two assays is presumably due to the presence of subunit I in the replicase tetramer (16).

Several laboratories have recently reported the isolation of an inhibitory protein, “i” factor, from the ribosomal wash of E. coli (5-7) with similar properties in initiation complex formation and translation of phage RNA as those of subunit I isolated in this laboratory. Kamen et al. (20) found that purified “i” factor could replace subunit I in the reconstitution of the intact Qβ replicase. The enzyme lacking subunit I was inactive with Qβ RNA plus strands but active with Qβ RNA minus strands or poly(C) as template.

Further studies from this laboratory on pure subunit I have shown that the inhibition of translation is extremely specific for the type of mRNA used, and results from the formation of a ribosome-subunit I complex (21).

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REFERENCES
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