Purification and Properties of Initiation Factor IF-3 from Caulobacter crescentus*

(Received for publication, August 13, 1973)

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SUMMARY

A five-step procedure is described for the purification to homogeneity of initiation factor IF-3 from ribosomal washes of Caulobacter crescentus. The protein, C-IF-3, has a molecular weight of 24,000 to 25,000 and seems to consist of a single polypeptide chain. It has three in vitro activities. (a) It promotes ribosomal binding of N-formyl methionyl transfer RNA directed by phage messengers; (b) it dissociates 70 S ribosomes into 30 S and 50 S subunits; (c) it binds single-stranded natural and synthetic RNAs. The factor is analogous to, and exchangeable with Escherichia coli IF-3. However, E. coli ribosomes do not bind N-formyl methionyl transfer RNA with C. crescentus phage Ch5 RNA as messenger and, conversely, C. crescentus ribosomes do not form an initiation complex with coliphage MS2 RNA or late T4 RNA. C-IF-3, as well as E. coli IF-3, have a high affinity for single-stranded stacked polymers, e.g. polyadenylate. Deviation from this type of structure, whether toward a more organized one, e.g. polyadenylate-polyuridylate, transfer RNA, or toward unstacking, e.g. polyuridylate, has an adverse effect on binding. DNA, native and denatured, is not bound at C-IF-3 concentrations sufficient to bind polyribonucleotides. Binding of C-IF-3 to coliphage MS2 RNA provides some protection from degradation by RNase.

Studies with initiation factor IF-3† from Escherichia coli have demonstrated that this protein is required for ribosomal binding of natural mRNAs (1, 2; for review see Ref. 3). However, the mechanism by which ribosomes select proper sites on the messenger for correct initiation is not understood; neither is the mechanism by which IF-3 causes the binding of the messenger to ribosomes. In addition to this activity, IF-3 dissociates 70 S ribosomes into 30 S and 50 S subunits (dissociation factor activity) (4) and remains bound to the 30 S subunits (5-7). This provides a pool of 30 S subunits competent to accept mRNA.

Investigations on initiation of protein synthesis in prokaryotes have been carried out largely on the E. coli system programmed with RNAs from RNA coliphages or T4 RNA. In vitro studies with systems from bacteria other than E. coli (8-10) have already indicated some species-specific selectivity in the ability of ribosomes to bind and translate coliphage RNAs. There is, however, a limitation in studies using only one class of host-specific, i.e. coliphage, natural messengers. This prompted us to develop an amino acid-incorporating system from Caulobacter crescentus programmed with a C. crescentus phage RNA (11). This system was selected because of the availability of the C. crescentus RNA phage, φCb5, which is similar in physical properties (12, 13) and analogous in genetic content (12) to RNA coliphages. RNA phages, other than coliphages, are known only for Pseudomonas aeruginosa (14) and for the Caulobacter group (15). The two parallel in vitro systems with species-specific mRNAs (E. coli-coliphage RNA and C. crescentus-φCb5 RNA) provide a model for investigations on the specificity of components—ribosomes, initiation and interference (16, 17) factors—responsible for the correct binding and translation of the messenger, and may provide new insights into the mechanism of ribosome-messenger recognition. We have previously shown (11) that the ability to translate phage messengers by these two systems depends upon the presence of the corresponding host 30 S subunit; the source of the initiation factors and the 50 S subunits is inmaterial.

In this communication we focus on the properties of initiation factor IF-3 from C. crescentus (C-IF-3). First, we describe a relatively simple procedure for the preparation of homogeneous C-IF-3. The pure protein displays three in vitro activities. (a) It promotes ribosomal binding of fMet-tRNA directed by phage messengers, (b) it has DF activity, and (c) it has RNA-binding activity. The factor is analogous to, and exchangeable with E. coli IF-3. However, E. coli ribosomes do not bind fMet-tRNA if φCb5 RNA is used as messenger and, conversely, C. crescentus ribosomes do not bind fMet-tRNA with coliphage MS2 RNA or T4 RNA as messengers. The binding of C-IF-3 to a number of natural and synthetic polynucleotides was examined. Efficient binding does not appear to be sequence-specific but requires a rather unique polynucleotide conformation. C-IF-3, as well as E. coli IF-3, have a high affinity for single-stranded stacked polymers, e.g. poly(A). Any deviation from this type of structure, whether in the direction of higher...
organization, e.g. poly(A-U), tRNA, or in the direction of unstacking, e.g. poly(U), has an adverse effect on binding. DNA, both native and denatured, is not bound at C-IF-3 concentrations sufficient to bind polyribonucleotides. Binding of C-IF-3 to oligophase MS2 RNA provides some protection from degradation by ribonuclease A.

EXPERIMENTAL PROCEDURES

Materials

C. crescentus Cb13 cells and phage Cb5 were kindly provided by Dr. L. Shapiro (Albert Einstein College of Medicine). E. coli Q13 and MR6000 were laboratory strains. [14C]Met-tRNA (312 mCi per mmole) and [14C]methionine (240 mCi per mmole) were purchased from New England Nuclear; E. coli W tRNA from Miles; polyethylene glycol 6000 to 7500 from Matheson-Coleman; ribonuclease A (RASE) from Worthington; all other chemicals were reagent grade.

Cb5 RNA was isolated as previously described (13); 70 to 80% of the RNA sedimented at 31 S. MS2 RNA was isolated as described by Weissmann and Feix (18). About 80 to 90% of the RNA sedimented at 29 S. Low T4 RNA, obtained by the procedure of Dautz and Hall (19), was kindly provided by Dr. S. Lee-Huang of this Department.

The following labeled polynucleotides were used in the RNA binding assay. [14C]poly(A) (1.2 mCi per mmole), [14C]poly(U) (1.0 mCi per mmole), and [3H]poly(U) (10 mCi per mmole) were从Miles; they were diluted with unlabeled polynucleotides and reprecipitated from 1 m KCl solutions with one volume of ethanol to give the following final specific radioactivities: [14C]poly(A), 1.050 cpm per mg; [14C]poly(U), 1.950 cpm per mg; [3H]poly(U), 6,500 cpm per mg; E. coli W [14C]RNA (Miles), 11,200 cpm per mg; MS2 [3H]RNA (16,100 cpm per mg) and T4[3H]DNA (45,600 cpm per mg) were kindly provided by Dr. M. C. Schneider and Dr. M. Rush of this Department. Formaldehyde treatment of MS2 [3H]RNA was as described by Lodish (20). [14C]-Labeled 16S and 16S ribosomal RNAs (1,240 and 1,320 cpm per mg, respectively) were obtained according to Stanley and Boek (21) from E. coli Q13 cells grown with 1 μCi of [14C]uracil.

Methods

Growth of Cells—C. crescentus cells were grown in a 180-liter vat fermentor, or in 16-liter carboys, at 30°C in a medium containing 0.2% bactopeptone, 0.1% yeast extract, and 0.05% MgSO₄ • 7H₂O in tap water. Cells were chilled and collected at mid-log phase by methods analogous to those established for E. coli (1).

Preparation of Ribosomes—C. crescentus ribosomes were obtained by the procedures analogous to those established for E. coli (1). Unfractionated ribosomes were washed with the standard buffer containing 0.5 mM NH₄Cl and 0.01 mM dithiothreitol instead of mercaptoethanol (standard buffer). Cells grown in the metal fermenter were used as a source of initiation factors; the ribosomes were more active when prepared from cells grown in carboys. E. coli cells were grown as described (1).

Preparation of Ribosomes—C. crescentus ribosomes were obtained by methods analogous to those established for E. coli (1). Unfractionated ribosomes were washed with the standard buffer (containing 0.5 mM NH₄Cl and 0.01 mM dithiothreitol instead of mercaptoethanol) for 12 hours, pelleted, and washed twice in the same buffer containing 1.0 mM NH₄Cl. They were stored in small aliquots in standard buffer containing 50% glycerol (v/v) at -70°C.

Amino Acid Incorporation Assay—C-IF-3 activity was assayed with either Cb5 RNA or MS2 RNA as messengers using C. crescentus or E. coli ribosomes, respectively. The assay mixture (0.06 ml) contained Tris-HCl buffer, pH 7.8, 60 mM NaCl, 60 mM ATP, 1.2 mM GTP, 0.2 mM phosphocreatine, 17 mM creatine kinase, 1 μM magnesium acetate, 10 mM or 14 mM with C. crescentus or E. coli ribosomes, respectively; dithiothreitol, 0.5 mM; E. coli W tRNA, 10 μg; [14C]lysine (specific radioactivity, 25 or 50 mCi per mmole); 0.1 mM; the remaining unlabeled amino acids, 0.1 M final concentration; 1.0 mM NH₄Cl-washed ribosomes, C. crescentus or E. coli, 2.5 A₂₆₀ units; E. coli Q13 150 μg × 10⁻⁶ g supernatant, about 120 μg of protein (this supernatant was used throughout as a source of aminoacyl-tRNA synthetases, etc., since it was more active than the corresponding E. coli fraction); Cb5 RNA or MS2 RNA, 0.6 to 0.8 A₁₀₀ unit; C-IF-3, 0.05 to 0.1 μg; C. crescentus C-IF-2a (homogeneous protein, molecular weight 84 × 10⁶), 0.1 to 0.3 μg, or E. coli IF-2 (purified IF-2, Ref. 22), 1.0 to 2.0 μg with C. crescentus or E. coli ribosomes, respectively; E. coli IF-1 (purified IF-1, Ref. 1), 1.0 to 1.5 μg. Initiation factor IF-1 was used to supplement only E. coli ribosomes because NH₄Cl-washed or run-off C. crescentus 70 S and 30 S ribosomes show no requirement for IF-1 (23). Incubation was for 20 min at 37°C. E. coli initiation factors were kindly provided by Drs. S. Lee-Huang, R. Mazumder, and S. Sabol of this laboratory.

[14C]Met-tRNA Binding Assay—The samples (0.05 ml) contained 50 mM Tris-HCl, pH 7.4; 50 mM NH₄Cl; 5.0 mM magnesium acetate; 0.2 mM GTP; 0.5 mM dithiothreitol; 1.0 A₂₆₀ unit of mRNA, as specified; 15 pmoles (6000 cpm) of [14C]Met-tRNA (32 pmols per 1.0 A₂₆₀ unit) and 2.0 A₂₆₀ units of E. coli or C. crescentus ribosomes, as specified; C. crescentus C-IF-2a, 0.1 to 0.3 μg, or E. coli IF-2, 1.0 to 2.0 μg with C. crescentus and E. coli ribosomes, respectively; E. coli IF-1, 1.0 to 1.5 μg, only in assays with E. coli ribosomes; C-IF-3, 0.5 to 0.8 μg, or E. coli IF-3, 1.2 to 2.0 μg. Incubation was for 10 min at 37°C. Ribosomal binding was determined by the Millipore filtration assay (24).

The preparation of [14C]Met-tRNA was described previously (25). Ribosome Dissociation (DF Activity) Assay—The samples (0.125 ml) in standard buffer, except that magnesium acetate was 0.005 M, contained E. coli MR6000 or C. crescentus 1.0 mM NH₄Cl-washed ribosomes, 1.0 A₂₆₀ unit; and C-IF-3 as indicated. Samples were incubated for 10 min at 37°C without C-IF-3; this procedure increases the ratio of 70 S to 30 S + 50 S ribosomes. The factor was added and samples were incubated for 15 min at 37°C. Control samples without factor were processed identically; 0.1 ml was layered on 5 ml of a 5 to 20% sucrose gradient in the same buffer and centrifuged at 4°C for 90 min at 42,000 rpm in the Spinco SW 50.1 rotor. Gradients were analyzed in an ISCO analyzer with a 10-mm light path flow cell. The proportion of 70 S, 50 S, and 30 S ribosomes was computed from the areas under the peaks; 30 μg of freshly prepared Step 5 C-IF-3 produced 85 to 95% dissociation under these conditions and this was taken as a relative DF activity. To express the results of Fig. 3 and Table 4 as relative DF activity, the specific activity of 1.0 A₂₆₀ was chosen by guest on October 15, 2017 http://www.jbc.org/ Downloaded from

RESULTS AND DISCUSSION

Purification of C-IF-3

C-IF-3 activity was assayed as described under "Methods" with Cb5 RNA and MS2 RNA as messengers using C. crescentus or E. coli ribosomes, respectively. Purification was also followed by SDS gel electrophoresis of steps 3 to 5 protein (Fig. 1). 1.0 M Ribosomal Wash (Step 1) All operations were carried out at 0−4°C. One kilogram of frozen cells was thawed, suspended in standard buffer to a volume of 2.0 liters and broken in a pressure cell under 7000 p.s.i. Deoxyribonuclease I (200 μg) was added, and the extract was centrifuged three times for 30 min
Origin at top, anode at bottom. Samples (0.08 ml) were layered trailing edge of the tracking dye (bromphenol blue) reached the bottom of the column (Gels A, B, and C, column length 7.2 cm); sodium phosphate buffer, pH 7.2, in the presence of 0.170 SDS. and pelleted. The resulting ribosomal wash was used as the at 30,000 X g in a Sorvall GSA rotor until a clear supernatant massie brilliant blue (0.25%) and destained in 7.5% acetic acid Step 5, 25 rg; E, Step 5, 15 lg.

at 30,000 X g in a Sorvall GSA rotor until a clear supernatant (S-30) was obtained. This was spun for 12 hours at 40,000 rpm in Spinclo Ti 60 rotors. The ribosomal pellet was washed for 12 hours in standard buffer containing 0.5 M NH₄Cl and pelleted at high speed as above. The 0.5 m NH₄Cl wash was used as the source of C-IF-2 (23). The ribosomal pellet was washed again for 12 hours in standard buffer containing 1.0 M NH₄Cl and pelleted. The resulting ribosomal wash was used as the source of C-IF-3.

Ammonium Sulfate Fractionation (Step 2)—The 1.0 M NH₄Cl ribosomal wash was fractionated with solid ammonium sulfate. The precipitate between 45 and 80% saturation contained over 90% of C-IF-3 activity. The protein pellet obtained after a 15-min centrifugation at 10,000 rpm in the Sorvall centrifuge was suspended in and dialyzed against two changes of a buffer containing 0.05 M NH₄Cl, 0.05 M Tris-HCl (pH 7.4), 5 X 10⁻⁴ M EDTA, 5 X 10⁻⁴ M dithiothreitol, and 10% glycerol (w/v) (Buffer Aₐo) for 12 hours.

Phosphocellulose Chromatography (Step 8)—The entire Step 2 dialysate (250 ml) was layered on a column (0.9 X 25 cm) previously equilibrated in buffer Aₐo. The column was washed with 50 ml of Buffer Aₐo followed by Buffer Aₐo until Aₐo of the effluent was near zero. Over 90% of the protein layered was removed in the wash. A linear gradient of NH₄Cl from 0.1 to 0.6 m in 160 ml of Buffer A was applied, and 2-ml fractions were collected. C-IF-3 elutes at 0.37 m NH₄Cl. The active fractions were pooled, dialyzed against Buffer Aₐo containing 50% polyethylene glycol (w/v).

DEAE-Sephadex Chromatography (Step 4)—The material from Step 3 (6 mg in 1 ml) was layered onto a column (0.9 X 10 cm) previously equilibrated in Buffer Aₐo. The column was washed with 25 ml of Buffer Aₐo followed by 25 ml of Buffer Aₐo containing 0.5 M NH₄Cl and eluted with the same buffer. The active fractions (6 ml) were pooled and concentrated in Buffer Aₐo containing 6 m urea and 50% polyethylene glycol (w/v). SDS gel electrophoresis of Step 4 protein demonstrates that a single contaminant is present in addition to the major band of C-IF-3 (Fig. 1). Step 4 C-IF-3 is free of an endonuclease activity which copurifies with the factor up to Step 3 (Fig. 2). The endonuclease is removed in the Aₐo column wash.

Sephadex G-100 Chromatography in 6 m Urea (Step 5)—Step 4 protein (3.6 mg in 0.9 ml) was layered on a column (0.9 X 65 cm) previously equilibrated with Buffer Aₐo containing 6 m urea and eluted with the same buffer. Exposure to this concentration of urea for up to 24 hours, does not inactivate C-IF-3. As shown in Fig. 3, C-IF-3 appears at a volume greater than the void volume and the incorporating activity with MS2 RNA and Ch5 RNA coincides with DF activity and RNA-binding activity. Note that C-IF-3 promotes the translation of both phage messengers by the corresponding host ribosomes; none of the phage messengers is translated by non-host ribosomes. SDS gel electrophoresis of Step 5 C-IF-3 reveals a single band (Fig. 1D). The active fractions were pooled, dialyzed against Buffer Aₐo containing 50% glycerol (v/v), and stored in small aliquots at -70°. Under these conditions, Step 5 C-IF-3 has a biological half-life of approximately 2 months, whereas Step 2 and Step 3 protein can be stored for many months without loss of activity. In all experiments reported in this paper, Step 5 C-IF-3 of specific activity not below 30% of the original was used.

Fig. 1. Polyacrylamide gel electrophoresis of C-IF-3 in 10 mm sodium phosphate buffer, pH 7.2, in the presence of 0.1% SDS. Samples (0.08 ml) were layered on 6.5% gels and run for about 6 hours at 5 ma per gel until the trailing edge of the tracking dye (bromphenol blue) reached the bottom of the column (Gels A, B, and C, column length 7.2 cm); in Gels D and E, the column length was 9.0 cm, and samples were run until the tracking dye moved either three-quarters (D) or halfway (E) through the column. Gels were stained with Coomassie brilliant blue (0.2%) and destained in 7.5% acetic acid.

Fig. 2. Removal of endonuclease during the purification of C-IF-3. Samples (0.125 ml) containing 1.2 Aₐo unit of MS2 RNA in standard buffer were incubated for 10 min at 37°, layered on linear sucrose gradients (5 to 20% in same buffer), and centrifuged for 90 min at 65,000 rpm in the Spinco SW 65 rotor at 4°. Gradients were analyzed in an ISCO analyzer with a 10-mm light path cell. A, control MS2 RNA; B, with 5 µg of Step 3 C-IF-3; C, with 10 µg of Step 4 C-IF-3. In a parallel assay with MS2 RNA, incubation with Step 3 C-IF-3 did not yield ethanol-precipitable material. This result is in agreement with the sucrose gradient profile of degradation and demonstrates the endonucleolytic nature of the cleaving enzyme(a).
and offers certain advantages over previous methods (e.g. Refs. 38). Amino acid incorporation assay with MS2 RNA and E. coli ribosomes; (c) MS2 RNA binding is similar to those obtained for E. coli IF-3 in an analogous reaction. The absolute values for the specific activity of Step 5 C-IF-3 are presented in Table I. For assay of DF activity and RNA-binding activity, fractions were dialyzed (Fractions 18 and 19 were pooled) against Buffer A100; (b) relative DF activity with C. crescentus 70 S ribosomes, Δ—Δ; virtually identical results were obtained with E. coli ribosomes (4, 28). Washing ribosomes with 0.5 M \( \text{NH}_4\text{Cl} \) before applying the 1.0 M \( \text{NH}_4\text{Cl} \) buffer for the extraction of C-IF-3 results in very small losses of activity and removes significant amounts of contaminating proteins. The use of phosphocellulose as the first ion exchange step in the procedure avoids the time-consuming separation of large amounts of protein on an anionic exchanger since it quickly removes over 90% of contaminants. Moreover, in contrast to the homogeneous factor, Step 3 protein (post-phosphocellulose) is fairly stable on storage and can be purified through Steps 4 and 5 in a short time. The results of Fig. 2 point to the importance of screening for nuclease activity during the purification of components to be used in protein synthesizing systems. In fact, crude extracts and Step 3 C-IF-3 promote a low level of \(^{[35}S\) incorporation with either Cb5 RNA and E. coli ribosomes or MS2 RNA and C. crescentus ribosomes. This most probably reflects some unspecific translation of RNA fragments for it occurs also when partially degraded phage RNAs are used as messengers. This is not the case with Step 4 C-IF-3.

Properties of C-IF-3

**Molecular Weight**—The molecular weight of C-IF-3 determined by SDS gel electrophoresis and gel filtration (see “Methods”) was 25,000 and 24,000, respectively. This indicates that C-IF-3 is a single polypeptide chain. C-IF-3 appears to be somewhat larger than E. coli IF-3 (4, 28).

\[^{[35}S\text{Met-tRNA Binding Directed by MS2 RNA, Cb5 RNA, and Late T4 RNA Experiments on the ribosomal binding of \(^{[35}S\text{Met-tRNA directed by three different types of mRNA are presented in Table II. With either E. coli or C. crescentus IF-3, MS2 RNA and late T4 RNA stimulate the binding to E. coli but not to C. crescentus ribosomes, whereas, Cb5 RNA stimulates \[^{[35}S\text{Met-tRNA binding to C. crescentus but not to E. coli ribosomes. These results show the same messenger specificity and IF-3 interchangeability as in the amino acid incorporation assay (Fig. 3 and Ref. 11). We have shown elsewhere that ribosomal specificity for the translation of MS2 RNA and Cb5 RNA resides in the corresponding host 30 S subunit (11); the source of the 50 S subunits is immaterial. We have also shown that C-IF-3 promotes the correct translation of MS2 RNA by E. coli ribosomes as judged by SDS gel electrophoresis of phage specific proteins synthesized in vitro (11).**

**DF Activity**—DF activity assayed in column fractions at Step 5 of purification overlaps with the amino acid incorporation activity (Fig. 3). Pooled Step 3 and Step 4 fractions were also assayed, showing qualitatively a substantial enrichment of DF activity with respect to Step 1 and Step 2 proteins. This indicates that, as with E. coli IF-3, C-IF-3 has probably a dual

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**Table I**

Summary of purification of C-IF-3

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume</th>
<th>Protein</th>
<th>Units</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 1.0 m NH(_4)Cl wash</td>
<td>ml</td>
<td>mg</td>
<td>(10^{-11})</td>
<td>units/mg</td>
</tr>
<tr>
<td>2. (NH(_4)SO(_4)) fractionation</td>
<td>ml</td>
<td>mg</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3. Phosphocellulose chromatography</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4. DEAE-Sephadex chromatography</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5. Sephadex G-100 chromatography in 6 M urea</td>
<td>-</td>
<td>-</td>
<td>2.1</td>
<td>1.78</td>
</tr>
</tbody>
</table>

* One unit = 0.1 nmole of \(^{[35}S\text{Met-tRNA incorporated under standard assay conditions with E. coli ribosomes and MS2 RNA (see "Methods").}

* From 1.1 kg of frozen cells.

* An endonuclease present up to Step 4 (see text and Fig. 2) rapidly degrades phage RNA thus interfering with the standard C-IF-3 assay.

**Table II**

Ribosomal binding of \(^{[35}S\text{Met-tRNA}}

Composition of samples and conditions of assays were as described under "Methods." Blank values without IF-3 (0.2 to 0.4 pmole with Cb5 and MS2 RNA, and 0.7 to 0.8 pmole with late T4 RNA) were subtracted.

<table>
<thead>
<tr>
<th>Ribosomes</th>
<th>IF-3</th>
<th>Cb5 RNA</th>
<th>MS2 RNA</th>
<th>Late T4 RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. crescentus</td>
<td>2.1</td>
<td>&lt;0.05</td>
<td>-0.1</td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td>1.6</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td>&lt;0.05</td>
<td>3.1</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td>C. crescentus</td>
<td>0.06</td>
<td>2.4</td>
<td>2.2</td>
<td></td>
</tr>
</tbody>
</table>
function in protein synthesis. The DF activity of C-IF-3 is not species-specific (Fig. 3).

**RNA-binding Activity**—E. coli IF-3 is known to bind phage RNA and synthetic oligonucleotides of the type ApUpG(pA)n (4, 29). With labeled RNA, this binding can be conveniently measured by the retention of label on Millipore filters. Fig. 3 shows that peaks of amino acid incorporation activity, DF activity, and RNA-binding activity of Step 5 C-IF-3 overlap. Furthermore, all three activities decrease upon storage of homogeneous C-IF-3 or after repeated freezing and thawing (Table III). Inactivation is even more pronounced in the DF- and RNA-binding assays for in these two, in contrast to the amino acid incorporation assay, C-IF-3 acts stoichiometrically rather than catalytically. There is no change in the SDS gel electrophoresis pattern of aged Step 5 preparations (specific activity in the amino acid incorporation assay decreased about 90% from the original 1200). These observations suggest that the RNA-binding activity of C-IF-3 is not an unspecific property of C-IF-3 as a basic protein but is closely related to its two other activities—DF and ribosomal binding of mRNA. This prompted us to examine more closely the binding of C-IF-3 to selected polynucleotides, natural and synthetic, at various ionic conditions and temperatures. It was previously noted that E. coli IF-1, although more strongly basic than IF-3, has no RNA-binding properties (4).

The binding at 0°C of C-IF-3 to poly(A), poly(U), poly(A·U), and poly(A·2U) in sodium phosphate buffer and in Tris-HCl buffer containing Mg²⁺ is shown in Fig. 4, A and B. In the phosphate buffer there is good binding to the single-stranded stacked poly(A) helix (30) and almost no binding to poly(U) which, under these conditions, exhibits a nearly unstacked, random coil structure (31). The situation is reversed in the Mg²⁺-containing Tris-HCl buffer (Plate B) where poly(A) binding is almost nil and poly(U) binding increases considerably. These results suggest that binding of IF-3 is not base-specific but depends rather on certain structural features of the polynucleotides. The increase in poly(U) binding apparently reflects an increased affinity of IF-3 to poly(U) when the polynucleotide is in an ordered state as is the case in Mg²⁺-containing Tris-HCl buffer (32, 33). The drop in poly(A) binding is less clear. Mg²⁺ may compete with IF-3 for binding sites or poly(A) may attain a more compact structure which prevents binding; the latter alternative is more in line with subsequent experiments. The binding of IF-3 to the poly(A·U) double-stranded complex in sodium phosphate buffer (Plate A) is significantly less than that of poly(A), and the binding of the triple-stranded poly(A·2U) is still less. The change in ionic conditions has little effect on the structure of the double-stranded and triple-stranded complexes, and, accordingly, there is little change in IF-3 binding. Some decrease in binding in the presence of Mg²⁺ may reflect a partial transition to a triple-stranded complex or to a more compact structure.

The experiments with synthetic polymers show that secondary and tertiary structure play a critical role in the IF-3 binding reaction. IF-3 has little affinity for highly ordered helices and even less for unstacked polynucleotides but binds efficiently, albeit in competition with Mg²⁺, to stacked single-stranded helices. These conclusions are partially borne out by experiments with natural polynucleotides carried out under the same conditions, although a more complex pattern of interaction between IF-3 and RNA emerges. As seen in Fig. 5, A and B, IF-3 has little affinity for tRNA and still less in the presence of Mg²⁺. This is in agreement with the observation that ordered structure has an adverse effect on binding. On the other hand, there is good binding to MS2 RNA in the presence of Mg²⁺ which is drastically reduced in sodium phosphate buffer. 16S and 23S ribosomal RNAs behave similarly, but binding is less efficient in the Mg²⁺-containing buffer and the decrease in binding in sodium phosphate buffer is less pronounced. There is virtually no binding to DNA and, significantly, to denatured DNA under the conditions of the assay. However, at very high concentrations of IF-3 (37.5 μg/0.1 ml of assay sample), considerable binding of DNA was obtained (not shown) indicating that unspecific effects occur under these conditions.

The experiments with single-stranded polynucleotides shown in Fig. 5 indicate that efficient IF-3 binding requires a unique type of structure. Certain binding sites on MS2 RNA become available for interaction when a specific Mg²⁺-induced conformation of the RNA molecule is attained. To some extent this holds for rRNAs as well. The results with synthetic and natural polynucleotides suggest that efficient binding takes place at unpaired, stacked regions of the RNA.

**Table III**

<table>
<thead>
<tr>
<th>Conditions of assay</th>
<th>Amino acid incorporation (specific activity in standard assay)</th>
<th>Relative DF activity</th>
<th>MS2 PHIRA binding activity (C-IF-3 in standard binding assay required for the retention of 50% of input cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freshly prepared Step 5 protein</td>
<td>1100</td>
<td>1.0</td>
<td>0.9</td>
</tr>
<tr>
<td>After 2 months at -70°C</td>
<td>480</td>
<td>0.2</td>
<td>5.0</td>
</tr>
<tr>
<td>After multiple thawing and freezing</td>
<td>Not detectable</td>
<td>Not detectable</td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 4.** Binding of C-IF-3 to synthetic polynucleotides. A, in 20 mM sodium phosphate buffer, pH 7.2; B, in 20 mM Tris-HCl buffer, pH 7.4, containing 10 mM magnesium acetate. Poly(A), ○—○; poly(A·U), ●—●; poly(A·2U), □—□; poly(U), △—△. Conditions of assay and specific radioactivities are given under "Experimental Procedures." [³⁵S]Poly(A) and [³¹P]poly(U) were used to prepare the complexes.
In further experiments we examined the Mg$^{2+}$ dependence of IF-3 binding to MS2 RNA, formaldehyde-treated MS2 RNA and 23 S ribosomal RNA (Fig. 6). A high level of binding to MS2 RNA is seen even at 1 mM Mg$^{2+}$. The extended binding plateau (up to about 10 mM Mg$^{2+}$) indicates that at moderate concentrations Mg$^{2+}$ does not compete effectively for C-IF-3 binding sites; however, at high Mg$^{2+}$ concentrations (about 20 mM) a more compact structure of the RNA may inhibit the formation of the complex with IF-3. The binding curves for the modified MS2 RNA and for 23 S RNA have similar shapes except that efficient binding starts at higher Mg$^{2+}$ levels. Note that in 20 mM Tris-HCl buffer used in this experiment, and in the absence of metal ions, there is much less binding than in 20 mM sodium phosphate buffer (cf. Figs. 5 and 6). Clearly, the presence of a metal counterion (or NH$_4^+$, not shown) is a necessary condition for C-IF-3 binding. This holds also for poly(A) binding which is greatly reduced in 20 mM Tris-HCl (not shown) as compared to the 20 mM sodium phosphate buffer (Fig. 4).

The temperature stability of C-IF-3-RNA complexes is shown in Fig. 7. The assays were carried out under optimal ionic conditions of binding for each polymer. Plateau levels of complexes formed at each temperature were obtained from time-course curves and plotted as a fraction of binding at 0°C. With poly(U) (20 mM Tris (pH 7.3), 10 mM Mg$^{2+}$) the complex is

**Table IV**

Protection by C-IF-3 of MS2 [H] RNA degradation by pancreatic ribonuclease

Composition of samples (in 20 mM Tris-HCl (pH 7.4)-10 mM magnesium acetate) and conditions of assay are as described under “Methods.” Incubation with C-IF-3 (3 μg per sample) was for 10 min at 0°C and for 5 min at 24°C.

| R.Nase added for 15 min at 0°C | Before addition of C-IF-3 | After addition of C-IF-3 | Complex retained
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete system</td>
<td>None</td>
<td>None</td>
<td>25,437</td>
</tr>
<tr>
<td>Experiment 1</td>
<td>None</td>
<td>None</td>
<td>263 (15%)</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>None</td>
<td>None</td>
<td>1,217 (4.8%)</td>
</tr>
<tr>
<td>Experiment 3</td>
<td>None</td>
<td>None</td>
<td>1,360 (5.2%)</td>
</tr>
<tr>
<td>Experiment 4</td>
<td>None</td>
<td>None</td>
<td>3,633 (14.2%)</td>
</tr>
<tr>
<td>R.Nase added for 10 min at 24°C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment 3</td>
<td>None</td>
<td>None</td>
<td>-18 (0%)</td>
</tr>
<tr>
<td>Experiment 4</td>
<td>None</td>
<td>None</td>
<td>685 (2.7%)</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>None</td>
<td>26 (0%)</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>None</td>
<td>45 (0%)</td>
</tr>
</tbody>
</table>

*a Net counts per min after subtraction of small blanks without C-IF-3 (about 140 to 180 cpm).*
virtually dissociated at 10°. Under these conditions the ordered state of poly(U) has a Tm of about 6° and, as in the experiments of Fig. 4, although at different ionic conditions, there is little binding to the random coil form of the polymer. The decreased complex formation with poly(A) (20 mM sodium phosphate, pH 7.3) with increasing temperature is gradual and again closely reflects structural changes of the polymer, namely the gradual extension of the stacked helix. With MS2 RNA (20 mM Tris (pH 7.4), 5 mM Mg2+ temperature-induced changes are similar to those with MS2 RNA except that the complex is less stable.

Binding of MS2 [3H]RNA to IF-3 affords partial protection from digestion with pancreatic ribonuclease (Table IV). This effect depends on the enzyme concentration and temperature. In Experiments 1 and 3 of Table IV, digestion by RNase is complete, i.e., it produces fragments which do not bind IF-3, addition of IF-3 prior to RNase affords about 3 to 4% protection. The protective effect is abolished by increasing the temperature (cf. Table IV Experiments 2 and 4). These observations may be helpful in finding some relationship between the RNA-binding activity of IF-3 and its role in ribosomal binding to natural mRNA. Sequence analysis of factor-protected fragments would allow a comparison with ribosome-protected sites (34).

Many of the RNA-binding experiments reported in this section were repeated with homogeneous E. coli IF-3. The results were virtually identical in all cases.

CONCLUSIONS

The isolation, in a homogeneous state, of a bacterial IF-3 other than E. coli made possible experiments which demonstrate the exchangeability of the two factors with respect to (a) phage RNA-directed ribosomal binding of the initiator aminoacyl-tRNA (b) phage RNA-directed protein synthesis (see also Ref. 11), (c) IF activity, and (d) RNA-binding activity. The phage RNAs used in this study were derived from two unrelated but physically and genetically analogous, E. coli and C. crescentus RNA phages. It follows that the two IF-3s do not discriminate between the phage messengers employed, nor are they selective toward different prokaryotic ribosomes. Specificity of messenger recognition in the two systems investigated resides in the 30 S ribosomal subunit (11). The RNA-binding experiments indicate that IF-3, whether E. coli or C. crescentus, has high affinity for certain structurally unique unpaired regions of the RNA which may correspond to initiation sites. These observations provide some clues to the mechanism by which IF-3 assists in the ribosomal binding of phage RNA, but it remains to be established what the relationship is between this activity and the RNA-binding activity, and what additional restrictions on RNA binding are imposed by the 30 S subunit itself (8, 11).

Acknowledgments—We thank Dr. S. Ochoa for his interest and comments on the manuscript and Drs. R. Mazumder and S. Lee-Huang for gifts of purified E. coli IF-1, IF-2, and IF-3. We thank Mr. W. Frazier for large scale growth of C. crescentus and E. coli Q13, Mr. G. Melders for preparation of phage MS2, and Miss M. DiPiazza for excellent technical assistance.

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