

Molecular Dissection of Translation Initiation Factor IF2

EVIDENCE FOR TWO STRUCTURAL AND FUNCTIONAL DOMAINS*

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By means of limited proteolysis of *Bacillus stearothermophilus* initiation factor IF2 and genetic manipulation of its structural gene, *infB*, we have been able to produce (or hyperproduce) and purify two polypeptide fragments corresponding to two structurally and functionally separate domains of the protein. The first is the G-domain (~41 kDa), which makes up the central part of the molecule and contains the conserved structural elements found in all GTP/GDP-binding sites of G-proteins. This domain is resistant to proteolysis in the presence of GTP or GDP, retains the capacity to interact with the 50 S subunit, binds weakly to the 30 S subunit, and displays ribosome-dependent GTPase activity with an approximately 2-fold higher K_m for GTP and the same V_{max} as compared with intact IF2. The second is the C-domain (~24 kDa), which corresponds to the COOH-terminal part of IF2 and constitutes an extraordinarily compact domain containing the fMet-tRNA binding site of IF2. In spite of its negligible affinity for the ribosomes, the C-domain weakly stimulates the ribosomal binding of fMet-tRNA, presumably by affecting the conformation of the initiator tRNA molecule.

and display a large degree of sequence homology in the COOH-terminal two-thirds of the molecule, but they hardly resemble each other in the NH₂-terminal part (Gualerzi *et al.*, 1990; Gualerzi and Pon, 1990). The conserved region of IF2 contains the characteristic sequence elements involved in the binding of guanosine nucleotides found in other G-proteins (Jurnak, 1985; March and Inouye, 1985). Aside from this GTP-binding site, however, nothing is known concerning the other active sites, of which there are at least three (*i.e.* the binding sites for the 30 S subunit, the 50 S subunit, and fMet-tRNA).

We had previously shown that, when subjected to digestion with three different proteases (*i.e.* trypsin, chymotrypsin, and Staphylococcal protease), *E. coli* IF2 yielded discrete fragments that were resistant to further proteolysis and that GTP or GDP was able to protect to some extent the molecule from cleavage (Pon *et al.*, 1985). In an extension of this work, we have recently identified the main tryptic cleavage sites in *B. stearothermophilus* IF2, as well as the sites of the molecule protected by the presence of GTP. Thus, we have shown that whereas the NH₂-terminal quarter of IF2 is rapidly degraded to yield fragments ≤ 5 kDa, the rest of the molecule is eventually cleaved to yield a fully resistant 24.5 kDa COOH-terminal fragment and an approximately 40-kDa fragment corresponding to the central region of the molecule. In the absence of GTP, an additional proteolytic cut in the middle of the consensus GTP-binding sequence cleaves this domain, producing two somewhat less resistant fragments of 23.5 and 17 kDa (Severini *et al.*, 1990).

In the present paper, both the 40- and 24.5-kDa fragments were first produced by preparative proteolysis and purified to homogeneity. Subsequently, we also manipulated the *B. stearothermophilus infB* gene to introduce specific deletions and to hyperproduce the above-mentioned IF2 fragments. All these fragments were tested *in vitro* for their activity in various partial reactions of the translation initiation pathway. Our results indicate that they correspond to two structurally and functionally distinct domains of the molecule.

MATERIALS AND METHODS

Buffers—The buffers used were the following: buffer A (Tris-HCl (pH 7.1), 20 mM; EDTA, 0.1 mM; 2-mercaptoethanol, 6 mM; glycerol, 10%), buffer B (Tris-HCl (pH 7.9), 20 mM; NH₄Cl, 100 mM; EDTA, 0.1 mM; 2-mercaptoethanol, 6 mM; glycerol, 10%), buffer C (Tris-HCl (pH 7.1), 30 mM; NH₄Cl, 30 mM; 2-mercaptoethanol, 1 mM; glycerol, 1.5%), buffer D (Tris-HCl (pH 7.6), 50 mM; NH₄Cl, 50 mM; KCl, 10 mM; MgCl₂, 13 mM; dithiothreitol, 0.5 mM), buffer E (Tris-HCl (pH 7.7), 15 mM; magnesium acetate, 7 mM; NH₄Cl, 50 mM; sucrose, 10%), buffer F (Tris-HCl (pH 8), 100 mM; NH₄Cl, 100 mM; magnesium acetate, 6 mM; 2-mercaptoethanol, 6 mM), and buffer G (Tris-HCl (pH 7.7), 50 mM; NH₄Cl, 100 mM; magnesium acetate, 7 mM; dithiothreitol, 1 mM).

General Procedures—Ribosomes and ribosomal subunits (from *E. coli* MRE600 and *B. stearothermophilus* 799), fMet-tRNA^{fMet}, NAcPhe-tRNA^{Phe} and initiation factors (all from *E. coli* MRE600)

Translation initiation factor IF2¹ is one of the three proteins required for initiation of mRNA translation in prokaryotes (Hershey, 1987; Gualerzi and Pon, 1990). Although IF2 is endowed with several properties and its biological activity is rather complex and not yet fully understood, its main function is to promote the binding of the initiator fMet-tRNA to the P-site of the ribosome in response to the initiation triplet of the mRNA.

The structural gene for initiation factor IF2 (*infB*) has been cloned, characterized, and sequenced in *Escherichia coli* (Sacerdot *et al.*, 1984), *Bacillus stearothermophilus* (Brombach *et al.*, 1986), *Streptococcus faecium* (Friedrich *et al.*, 1988), and *B. subtilis* (Shazand *et al.*, 1990).

Comparison of the primary structures of the corresponding proteins shows that they have approximately the same size

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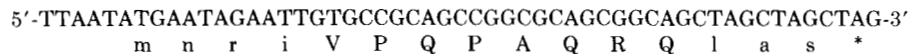
¹ The abbreviations used are: IF, initiation factor; EF, elongation factor; kb, kilobase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; NAcPhe, *N*-acetylphenylalanyl.

were prepared essentially as described (Ohsawa and Gualerzi, 1983; Pawlik *et al.*, 1981). Poly(AUG)-dependent and MS2 RNA-dependent ribosomal binding of fMet-tRNA, poly(U)-dependent binding of NAcPhe-tRNA, and *in vitro* translation directed by MS2 RNA or poly(U) were performed as previously described (Ohsawa *et al.*, 1983; Gualerzi *et al.*, 1989).

Electrophoreses on slabs of polyacrylamide containing SDS (SDS-PAGE) were carried out as described (Laemmli, 1970; Studier, 1973). Electroblotting of peptides and proteins, as well as automated amino acid microsequencing were performed as previously described (Severini *et al.*, 1990).

Overproduction of IF2—*B. stearothermophilus* IF2 was overproduced in *E. coli* K12ΔH1Δtrp cells harboring pIM401 (Brombach *et al.*, 1986) that had been constructed by placing the *B. stearothermophilus infB* gene behind the λ P_L promoter in pLcC2833 (Remaut *et al.*, 1983). Cell growth, induction, and conditions for the overproduction were as described (Brombach *et al.*, 1986).

Construction of an Expression Vector for the G-domain Overproduction—*B. stearothermophilus infB* gene cloned in pIM401 was interrupted by introduction of a SMURFT (Pharmacia LKB Biotechnology Inc.) linker into the PvuII site within its coding sequence; thus, a termination codon was introduced so that, upon induction, a shortened IF2 consisting of the first 514 amino acids was overproduced by cells bearing this construct. Subsequently, from this construct, the ~1.2-kb *BanI*-*ClaI* fragment was excised and subcloned into pEV2 (Crowl *et al.*, 1985) via *ClaI* sites and blunt-end ligation of filled-in *BanI* and *EcoRI* sites to yield an expression vector (pXP401G) that overproduces a polypeptide corresponding to the G-fragment with the following modifications to the sequence at the ends.



The amino acids indicated by lower case letters are those introduced by coding sequences derived from either pEV2 at the NH₂ terminus or the SMURFT linker at the COOH-terminus.

Hyperproduction and Purification of the G-fragment—*E. coli* pop3184 cells (a kind gift of Dr. R. Calogero, Sorin Biomedica, Saluggia, Italy) transformed with pXP401G were grown and induced as described for the hyperproduction of IF2 (Brombach *et al.*, 1986).

The high salt (1 M NH₄Cl) postribosomal supernatant was obtained from approximately 60 g of cells following the procedure described (Pawlik *et al.*, 1981). After 10-fold dilution with buffer A to reduce the NH₄Cl concentration to 100 mM, this fraction was loaded on a phosphocellulose column (3.5 × 14 cm) equilibrated with the same buffer containing 100 mM NH₄Cl. The progress of the purification of the G-fragment was followed by SDS-PAGE (12.5% acrylamide) as indicated above. The G-fragment, which was found in the flow-through and in the 350-ml wash fraction of this column, was then loaded on a DEAE-cellulose column (3.5 × 14 cm) equilibrated with buffer B. After a 350-ml wash with the same buffer, the column was eluted with a 1.5-liter NH₄Cl gradient (100–500 mM) in buffer B, collecting 10-ml fractions. The fractions containing the G-fragment, which was eluted within the first third of the gradient, were pooled, exhaustively dialyzed against buffer A containing 50 mM NH₄Cl, and loaded on a CM-cellulose column (3.5 × 30 cm) equilibrated with the same buffer. After washing with 300 ml of the same buffer, the column was eluted with a 3-liter NH₄Cl gradient (50–350 mM), collecting 20-ml fractions. The G-fragment eluting from the column in the initial part of the gradient was collected, concentrated, and finally purified by gel filtration on a Sephadex G-75 column equilibrated with buffer A containing 200 mM NH₄Cl. After concentration and dialysis against the same buffer, the G-fragment was stored at –80 °C.

Proteolysis of IF2 and Purification of the C-domain—Twenty-mg aliquots of IF2 were incubated for 30 min at 37 °C with 20 μg of trypsin (L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated) in 20 ml of buffer C containing 1 mM GTP. After addition of 4 mg of soybean trypsin inhibitor, the mixture was loaded on a 3 × 10-cm DEAE-cellulose column equilibrated with buffer A containing 100 mM NH₄Cl. After washing with 200 ml of the same buffer, the chromatographic column was eluted with a 600-ml NH₄Cl gradient

(100–400 mM) in buffer A, collecting 2.5-ml fractions. Fifty-μl aliquots of every fifth fraction were analyzed on SDS-PAGE, and the fractions containing the C-fragment were pooled. After reduction of the volume, the protein was further purified by gel filtration on a Sephadex G-50 superfine column equilibrated with buffer B containing 200 mM NH₄Cl. The fractions containing the C-fragment were detected electrophoretically and pooled. After concentration and exhaustive dialysis against the same buffer, the C-fragment was stored at –80 °C.

IF2- and Ribosome-dependent GTPase Activity—Unless otherwise specified, each incubation mixture contained, in 50 μl of buffer D, 240 pmol each of 30 and 50 S ribosomal subunits, 60 pmol of either IF2 or G-fragment, as well as GTP at the concentrations indicated in each experiment. To obtain this, prior to the reaction, a small amount of [γ-³²P]GTP (10 Ci/mmol) was mixed with nonradioactive GTP to yield fresh stock solutions of radioactive GTP. After determination of the specific activity, appropriate aliquots of these solutions were added to the reaction mixtures to attain the desired final concentrations of GTP. The mixtures were then incubated for 5 min at 37 °C, and the extent of hydrolyzed GTP was determined by isopropylacetate extraction in an acidic environment of the ³²P-dodecamolybdate complex as described by Parmeggiani and Sander (1981).

Binding of IF2 and IF2 Fragments to 30 and 50 S Ribosomal Subunits—The experiments were carried out essentially as previously described (Pon *et al.*, 1985), except that native, unlabeled protein was used in place of the *in vitro* labeled protein. Consequently, the unbound protein was quantified by a densitometric assay. Each incubation mixture contained, in 175 μl of buffer E, 280 pmol of protein (IF2, C-fragment or G-fragment) and increasing amounts (from 0–3 nmol) of *B. stearothermophilus* 30 or 50 S ribosomal

subunits. After a 5-min incubation at 11 °C, the samples were centrifuged for 60 min at 30 p.s.i. in a Beckman Airfuge. Seventy μl of supernatant were withdrawn from the top of each tube and subjected to SDS-PAGE analysis followed by densitometric determination of the amount of protein remaining unbound.

RESULTS

As mentioned above, the C- and G-fragments of *B. stearothermophilus* IF2 were originally obtained by standard chromatographic purification of preparative tryptic digests obtained in the presence of GTP. By this method, a high yield of very pure (>95%) C-fragment was reproducibly obtained, but the yield and purity (which never exceeded 90%) of the G-fragment were not entirely satisfactory. Thus, we decided to purify this fragment following hyperproduction by cells transformed with an expression vector carrying the appropriately manipulated segment of *B. stearothermophilus infB*. Later on, a similar genetic approach was used to produce the C-fragment also. Although we have used C- and G-fragments of IF2 prepared by both proteolysis and genetic engineering and in no circumstance found any difference in their structural and functional properties, it should be mentioned that the results shown here were obtained exclusively using C-fragment purified following preparative proteolysis and G-fragment purified from an extract derived from hyperproducing cells.

The electrophoretic purity of the C- and G-fragments used throughout this study is shown in Fig. 1. The NH₂-terminal sequence of the C-fragment was found to be SVK-TRVSLDDLFE. Thus, the C-fragment begins at Ser⁵²⁰ of *B. stearothermophilus* IF2 and, judging from its apparent molecular weight (26,500) determined by denaturing gel electrophoresis, should span through the COOH terminus of the molecule. In fact, if no amino acids have been lost from the COOH

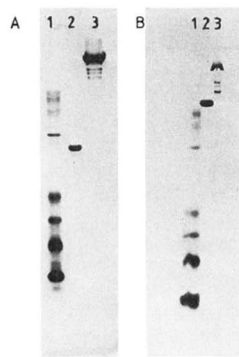


FIG. 1. Electrophoretic homogeneity of *B. stearotherophilus* IF2 fragments. A, C-fragment on 15% SDS-polyacrylamide gel; B, G-fragment on 12.5% SDS-polyacrylamide gel. Lanes 1, molecular weight markers: ovalbumin (43,000), α -chymotrypsinogen (25,700), β -lactoglobulin (18,400), lysozyme (14,300), bovine trypsin inhibitor (6,200), insulin (3,000); lanes 2, 12 μ g of C-fragment (panel A) or 14 μ g of G-fragment (panel B); lanes 3, *B. stearotherophilus* native IF2, 30 μ g (panel A) or 12 μ g (panel B). After electrophoresis carried out as described under "Materials and Methods," the polyacrylamide gels were stained with 0.1% Coomassie Blue (in 10% acetic acid and 50% methanol in water) and destained with 10% acetic acid and 10% methanol in water.

terminus, the chemical molecular weight of the C-fragment should be 24,544.

The NH₂- and COOH-terminal sequences of the G-fragment are MNRIVPQPAK- and -AQRQLAS, respectively, with the amino acid residues not underlined being introduced by the genetic manipulations described under "Materials and Methods." Thus, the G-fragment consists of 373 amino acid residues, 367 of which, from Val¹⁴⁸ to Leu⁵¹⁵, belong to the IF2 molecule. Both the apparent and chemical (*i.e.* from its deduced amino acid composition) molecular weights of the G-fragment are ~41,000.

From the structural point of view, both C- and G-fragments represent very compact, protease-resistant domains. In fact, even incubations for several hours of the C-fragment with trypsin or other proteases (*i.e.* Staphylococcal protease or chymotrypsin) does not result in a further degradation of this fragment (not shown). The G-fragment, on the other hand, though less resistant than the C-fragment, can withstand trypsinization for some time, at least in the presence of GTP (Fig. 2) before being cleaved into two subdomains (I and II) by a proteolytic cut occurring just in the middle of the GTP-binding region in correspondence with the Arg³⁰⁸-Ala³⁰⁹ bond (Severini *et al.*, 1990). The two subdomains are also relatively resistant to further proteolysis, but they are eventually degraded to smaller peptide products.

Both C- and G-fragments of IF2 were tested in various partial reactions of the initiation pathway, and their activity was compared with that of the intact molecule. As seen in Fig. 3, the C-fragment is able to interact with fMet-tRNA, as indicated by its capacity to protect the initiator tRNA from the spontaneous hydrolysis occurring in slightly alkaline Tris buffer. When subjected to a similar test, the G-fragment proved to be completely inactive, as was a mutated IF2 molecule missing a stretch of 132 amino acids within its C-domain (see the *hatched area* in Fig. 8). This molecule was obtained by deletion of a ~0.2-kb *Cla*I fragment from *B. stearotherophilus infB*.

Compared with native IF2, the affinity of the C-fragment for fMet-tRNA seems to be reduced by approximately 1 order of magnitude, at least judging from the protection experiments

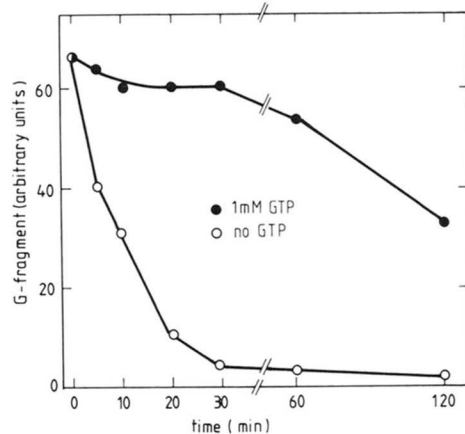


FIG. 2. Protection by GTP of G-fragment from further proteolysis. The experiment was carried out on an analytical scale (*i.e.* reducing by 100-fold the volume and the amounts of protein and trypsin) as described under "Materials and Methods" using purified G-fragment in place of IF2 and incubating the samples for the indicated times in the presence (●) or absence (○) of 1 mM GTP. Aliquots of the samples were then subjected to SDS-PAGE followed by densitometric scanning of the slabs.

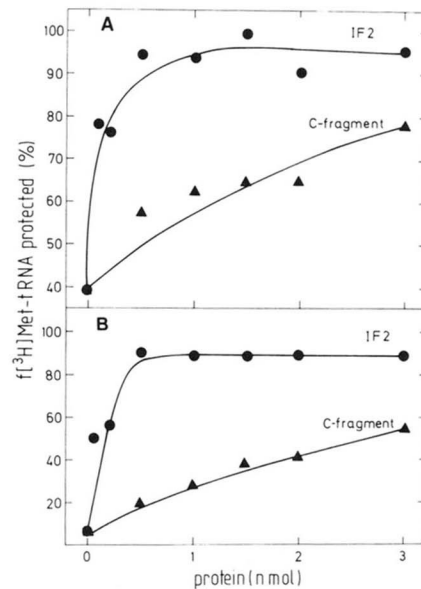


FIG. 3. Protection of fMet-tRNA from spontaneous hydrolysis by *B. stearotherophilus* intact IF2 and its C-fragment. The experimental protocol was essentially as described by Petersen *et al.* (1979). Each incubation mixture (90 μ l of buffer F) contained 50 pmol of f[³H]Met-tRNA^{fMet}, 1 mM GTP, and the indicated amounts of either IF2 (●) or C-fragment (▲). The incubation was carried out at 37 °C, and duplicate samples of 10 μ l were withdrawn at 0, 30, and 120 min for the determination of the remaining trichloroacetic acid-insoluble f[³H]Met-tRNA^{fMet}. Panel A, spontaneous hydrolysis after a 30-min incubation; panel B, spontaneous hydrolysis after 120 min.

of Fig. 3. However, a gel shift assay developed more recently² indicates a somewhat smaller difference in the binding affinity.

The C-fragment was also found to induce a small but reproducible stimulation of the ribosomal binding of fMet-tRNA (Fig. 4, A and B) and of its analogue NAcPhe-tRNA (Fig. 4C) in response to random poly(AUG) and poly(U), respectively. The stimulation of initiator tRNA binding was distinctly more pronounced with 70 S ribosomes (Fig. 4B)

² T. Suryanarayana and C. O. Gualerzi, unpublished observations.

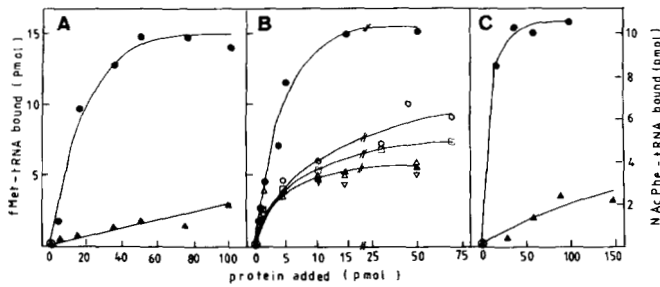


FIG. 4. Effect of IF2 and C-fragment of IF2 on the ribosomal binding of fMet-tRNA and NAcPhe-tRNA. Panel A, poly(AUG)-dependent binding of fMet-tRNA to 30 S ribosomal subunits in the presence of IF2 (●) or C-fragment (▲). Panel B, poly(AUG)-dependent binding of fMet-tRNA to 70 S ribosomes in the presence of IF2 (●) or C-fragment (▲) in a complete system, in the presence of IF2 in the absence of IF1 (□) or of GTP (○), and in the presence of C-fragment in the absence of IF1 (▽) or of GTP (△). Panel C, poly(U)-dependent binding of NAc¹⁴C-Phe-tRNA in the presence of IF2 (●) or C-fragment (▲). Binding of NAcPhe-tRNA was carried out essentially as described (Ohsawa and Gualerzi, 1983). The incubation mixtures for the binding of fMet-tRNA contained, in 50 μ l of buffer G, 5 μ g of random poly(AUG), 1 mM GTP, 20 pmol of [³H]Met-tRNA, 40 pmol each of IF1 and IF3, and 0.5 A_{260} units of *E. coli* 30 S ribosomal subunits (panel A) or 1.5 A_{260} units of *E. coli* 70 S ribosomes (panel B). In addition, each mixture contained the amounts of *B. stearoothermophilus* IF2 or C-fragment of IF2 indicated on the abscissa. After a 20-min incubation at 37 °C, the amount of bound aminoacyl-tRNA was determined by standard filtration onto nitrocellulose. Since both IF2 and C-fragment retain fMet-tRNA on nitrocellulose filters in the absence of ribosomes, this background was subtracted to calculate the ribosome-bound fMet-tRNA. This is easily done when the amount of protein is small, but it becomes increasingly difficult when very large amounts of protein are added. Consequently, the experimental points for the higher amounts of protein are somewhat less reliable than those obtained from the lower amounts.

than with 30 S subunits (Fig. 4A). The existence of two plateaus, with IF2 and with C-fragment, clearly visible in the experiment of Fig. 4B, reflects the fact that the position of the equilibrium is different under the two experimental conditions, because the ribosomal ligands are different (*i.e.* fMet-tRNA – IF2 *versus* fMet-tRNA – C-fragment, with the latter interacting with the ribosome only via the tRNA moiety). In similar tests, the G-fragment showed no stimulation of aminoacyl-tRNA binding and, when added to a system containing the C-fragment, did not improve the performance of the latter but actually abolished its stimulation of fMet-tRNA binding.

The stimulation of fMet-tRNA binding by the C-fragment is not affected by either IF1 or GTP and attains binding levels similar to those observed with the intact IF2 molecule in the absence of either GTP or IF1 (Fig. 4B). Also noteworthy is that, with respect to that of IF2, the stimulatory activity of the C-fragment is anomalous from several points of view: it can be seen exclusively with synthetic templates such as random poly(AUG) or poly(U) but not with MS2 RNA, and the fMet-tRNA bound to the ribosomes in the presence of the C-fragment, unlike that bound with IF2, does not react with puromycin (not shown). Finally, it is remarkable that the stimulation of fMet-tRNA and NAcPhe-tRNA binding is not sustained by any detectable affinity of this fragment for either 30 or 50 S subunits or 70 S ribosomes (see also below). All these unusual properties of the C-fragment-induced stimulation of fMet-tRNA binding could possibly provide important clues to the molecular mechanism of action of IF2 (see "Discussion").

The effect of the C-fragment in protein synthesis *in vitro* was also studied in a cell-free system programmed with MS2 RNA and with poly(U) at a low magnesium concentration in

the presence of NAcPhe-tRNA and the initiation factors IF1 and IF3. As expected, intact IF2 greatly stimulated translation in both systems; the C-fragment, on the other hand, was found to inhibit the background (*i.e.* IF2-independent) translation (not shown). Since this inhibition could be completely relieved by addition of fMet-tRNA or NAcPhe-tRNA, this inhibition could be attributed to the sequestering of the two aminoacyl-tRNAs in nonproductive complexes with the C-domain.

As to the G-fragment, the experiment of Fig. 2 has clearly demonstrated that this domain retains the capacity to interact with GTP. The experiments shown in Fig. 5 and 6 further show that the G-domain also retains the capacity to catalyze the ribosome-dependent hydrolysis of GTP. When the initial rate of GTP hydrolysis was measured with IF2 and G-fragment as a function of increasing GTP concentrations, the resulting double reciprocal plot showed straight lines (Fig. 6); this is a common finding with the other ribosomal GTPases EF-G and EF-Tu (Parmeggiani and Sander (1981) and references therein). From the results of Fig. 6, it can be calculated that, in the presence of ribosomes, the G-fragment catalyzes

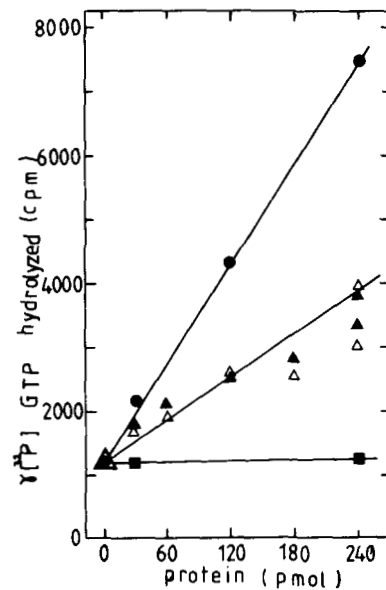


FIG. 5. Ribosome-dependent GTPase activity of IF2 and of its C- and G-fragments. The reaction (10 min at 37 °C) and the assay of GTP hydrolysis were carried out as described under "Materials and Methods," except that the amount of protein was varied as indicated in the abscissa. The reaction mixtures contained 0.1 mM [γ -³²P]GTP (1,515 cpm/nmol) and IF2 (●), G-fragment (▲), C-fragment (■), and both C- and G-fragments (△).

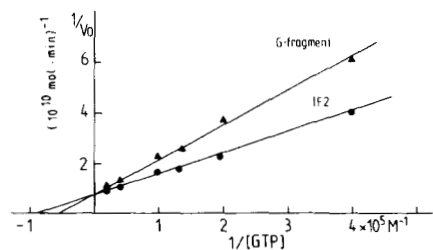


FIG. 6. Determination of K_m and V_{max} for ribosome-dependent GTPase activity of IF2 and G-fragment. The reactions were carried out as described under "Materials and Methods" in reaction mixtures containing the GTP concentrations indicated in the abscissa. The K_m values determined were 9 and 16 μ M for IF2 and G-fragment, respectively, and the V_{max} was 2.2 mol of GTP hydrolyzed/mol of protein/min for both IF2 and G-fragment.

GTP hydrolysis with essentially the same V_{max} ($2.2 \text{ mol} \cdot \text{mol}^{-1} \cdot \text{min}^{-1}$) as intact IF2 but with a slightly higher K_m for GTP ($16 \text{ versus } 9 \mu\text{M}$). The C-fragment, on the other hand, was found to be completely inactive in this test and, when added to a reaction mixture containing the G-fragment, did not produce any stimulation of the GTPase activity (Fig. 5). As seen from Fig. 5, the GTPase activity of the G-fragment is somewhat lower compared with that of intact IF2, and the difference seems to increase when increasing amounts of protein are added to a fixed amount of ribosomes. This behavior suggested to us that one of the possible reasons for the reduced GTPase activity of the G-domain could have been a reduced affinity of this fragment for the ribosome.

The interaction of the C-domain and the G-domain of IF2 with ribosomes was investigated by ligand-binding experiments carried out in the Beckman air-driven centrifuge (Airfuge) using *in vitro* labeled proteins (Pon *et al.*, 1985). These experiments showed that the C-domain was completely inactive in binding to either 30 or 50 S ribosomal subunits from both *E. coli* and *B. stearothermophilus*, whereas the G-domain was able to interact with all kinds of subunits but showed the unexpected property of binding better to the large subunit (not shown); however, since the *in vitro* labeling procedure used in our experiments could have caused a selective inactivation of the C-domain and/or of the 30 S binding site of the G-domain, we decided to repeat our experiments using a method that does not entail any chemical modification of the proteins under scrutiny (Fig. 7). Thus, after centrifuging reaction mixtures containing fixed amounts of "native" protein (*i.e.* IF2, C-fragment, or G-fragment) with increasing amounts of either 30 or 50 S ribosomal subunits, we analyzed quantitatively (*i.e.* by densitometry) the electrophoretically resolved, ribosome-unbound protein remaining in the upper part of the Airfuge tubes. As seen from the figure, the G-fragment, unlike IF2 (Weiel and Hershey, 1982; Pon *et al.*, 1985), indeed binds to the 50 S better than to the 30 S ribosomal subunit. The C-fragment, on the other hand, was again confirmed to be completely inactive in ribosomal binding (not shown). The above-described binding assay method, although free from the potential drawbacks of the chemical modification of the ligands, is unfortunately not suitable for an accurate determination of the association constants. In fact, due to the very large amounts of ribosomes needed to obtain a detectable binding, the solubility limit of the ribosome is approached. Under these conditions, nonspecific subunit-subunit interaction and the spillover of unsedimented subunits in the "supernatant" fraction of the Airfuge tube becomes a serious problem obscuring some G-fragment bind-

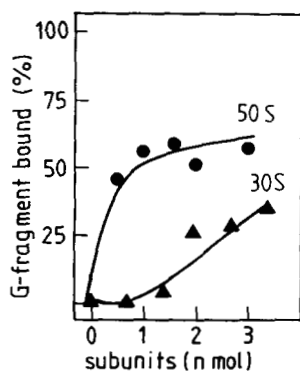


FIG. 7. Binding of G-fragment to *B. stearothermophilus* 30 and 50 S ribosomal subunits. The ribosomal binding of the protein was assayed by Airfuge (Beckman) centrifugation, essentially as previously described (Pon *et al.*, 1985).

ing. This can explain the probably artifactual tendency of the binding curve to indicate a plateau at about 60% bound G-fragment, which should therefore not be interpreted to mean that a large fraction of the G-fragment is inactive in ribosome binding. Nevertheless, since IF2 and the G-fragment gave nearly superimposable 50 S-binding curves under the conditions of the experiment of Fig. 6 (not shown), we can conclude that, compared with intact IF2, the G-domain has an approximately identical affinity (*i.e.* $K_a \approx 10^6 \cdot \text{M}^{-1}$) for the 50 S subunit and between 1 and 2 orders of magnitude lower for the 30 S subunit. Thus, the premise that the G-fragment binds to ribosomes, a corollary of its ribosome-dependent GTPase activity, and the above prediction that its affinity for the ribosome might be somewhat lower than that of native IF2, are both confirmed from the results of the binding experiments.

DISCUSSION

Prokaryotic IF2 contains at least four active sites: the binding sites for GTP, the 30 S subunit, the 50 S subunit, and fMet-tRNA. The identification of the GTP/GDP binding site is straightforward, due to the sequence identity with the homologous regions of elongation factors EF-Tu and EF-G; indeed, Vachon *et al.* (1990) recently demonstrated that a ~20 kDa fragment of *E. coli* IF2, obtained by shortening the corresponding *infB* gene, can bind GTP without displaying any other functional activity.

In this paper, we present a first attempt to localize and identify the active sites of IF2 and to gain a better insight into the structural organization of this protein. Our work originated from the observation that proteolysis of *E. coli* IF2 proceeds with a characteristic and reproducible pattern and that GTP affords protection of a specific cleavage site (Pon *et al.*, 1985). This work has recently been confirmed and extended using thermophilic IF2 which, owing to its overall greater stability and a higher affinity for its ligands, has in the meanwhile proven to be a molecule highly suitable for structural and functional studies (Brombach *et al.*, 1986; Severini *et al.*, 1990).

Thus, rather than using an indiscriminate fragmentation of the IF2 molecule, we favored the approach of the proteolytic (and later genetic) isolation of naturally occurring domains of the molecule that were then structurally and functionally characterized.

Taken together, our results indicate that IF2 contains at least two compact domains (the C-domain and G-domain) and a flexible, labile region (the NH₂-terminal region) (Fig. 8). Although these elements of the molecule must be structurally and functionally connected, as also suggested by the finding that the 30 S binding capacity of IF2 is nearly abolished following fragmentation of the molecule, the results allow us to attribute particular properties and functions to each region of IF2.

The C-domain—This domain constitutes an extraordinarily compact domain showing extensive resistance to proteolysis, even in the absence of ligands. It contains the fMet-tRNA binding site of IF2 and, in spite of its complete lack of affinity for ribosomes, is able to stimulate weakly the binding of NAcPhe-tRNA and of fMet-tRNA to both 30 S subunits and 70 S ribosomes. This stimulation is seen only in response to poly(U) or random poly(AUG), but not in the presence of MS2 RNA, however, and unlike that of IF2, is not increased by IF1 and GTP, and the ribosome-bound fMet-tRNA is not found in a puromycin-reactive site. Thus, in a ribosome complexed with either poly(U) or random poly(AUG), the chances that the ribosomal P-decoding site is occupied by a correct

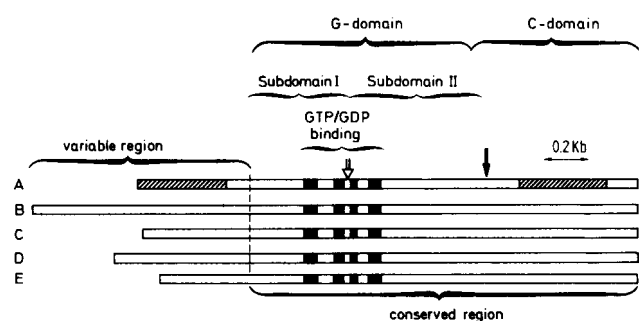


FIG. 8. Schematic representation of some relevant properties of the IF2 molecule and localization of its domains. The IF2 molecules and their regions of conserved and variable sequence are drawn to scale. A, *B. stearothermophilus*; B, *E. coli* α-form; C, *E. coli* β-form; D, *S. faecium*; E, *B. subtilis*. The four sequence elements involved in the binding of the guanosine nucleotides are indicated by the blackened areas. Trypsinolysis rapidly degrades the variable region and yields the G- and C-domains following the cleavage occurring in the position indicated by the black arrow. In the absence of GTP, the G-domain is cleaved by trypsin in the middle of the GTP-binding site (white arrow), yielding the two relatively resistant subdomains I and II. The hatched areas represent the deletions introduced by exonuclease Bal31 in the variable region and by deletion of a ~0.2-kb *Cla*I fragment in the C-domain.

triplet are high. In the case of MS2 RNA, however, owing to the more complex structural constraints of the template, also imposed by the existence of the Shine-Dalgarno interaction, some kind of rearrangement is probably required in order to place the specific initiation triplet in the decoding site (Taniguchi and Weissmann, 1979). Several data support this premise and the idea that IF2 participates in this process (Canonaco *et al.*, 1989).³ Thus, the properties of the C-domain are at least compatible with the hypothesis that the role of IF2 and its mechanism of action on the ribosome is much more complex than previously thought. Indeed, recent data support the notion that IF2 promotes a mutual rearrangement of the mRNA and initiator tRNA on the ribosome, resulting in a shift of the mRNA into its ribosomal binding channel and bringing the 3'-end of the fMet-tRNA in the ribosomal peptidyltransferase center (Canonaco *et al.*, 1989).⁴ The latter function depends on the hydrolysis of GTP. If all this is correct, it is not surprising that only the entire IF2 molecule and not the C-fragment, which is totally inactive in ribosomal binding and GTPase activity, can support these sophisticated functions.

The modest stimulation of the ribosomal binding of NAcPhe-tRNA and fMet-tRNA by the C-domain of IF2, on the other hand, can most likely be attributed to an effect of IF2 and probably of the C-fragment on the conformation of the anticodon stem and loop of initiator tRNA, which probably favors codon-anticodon interaction (Wakao *et al.*, 1989). Thus, while resting on the specific interaction that the C-domain of the molecule can establish with NH₂-blocked aminoacyl-tRNAs, this stimulation seems to uncover an ancillary property of IF2, perhaps contributing to the overall function of the factor.

The G-domain—This ~41 kDa domain makes up the central part of the molecule and contains the conserved structural elements found in all GTP-binding sites of G-proteins (Jurnak, 1985; March and Inouye, 1985). In the presence of GTP or GDP, this domain is relatively resistant to proteolysis, and seems to consist of two fairly compact subdomains (I and II)

whose further characterization has not yet been obtained, however. The G-domain retains the capacity to bind to the 50 S subunit with approximately the same affinity as native IF2 and to interact, albeit very weakly, with the 30 S subunit. The G-domain also retains the ribosome-dependent GTPase activity of IF2 displaying the same V_{max} and a slightly higher K_m for GTP as native IF2.

N-domain—Large deletions from the 5' terminus of IF2 (see Fig. 8), obtained following genetic manipulation of *infB* of both *E. coli* and *B. stearothermophilus* (Brombach *et al.*, 1986; Pon and Gualerzi, 1988), or following proteolysis of the factor (Cenatiempo *et al.*, 1987) did not result in the loss of any of the basic translational activities of IF2. This fact and the finding that the NH₂-terminal part of IF2 is not evolutionarily conserved and is very readily cleaved by proteases (Severini *et al.*, 1990) suggest that this region of the molecule has a loose and flexible structure and might have a yet unidentified, species-specific function probably not related to translation.

A preliminary account of this work has been presented elsewhere (Spurio *et al.*, 1989).

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³ R. Brandt and C. O. Gualerzi, unpublished observations.

⁴ R. Brandt, A. La Teana, and C. O. Gualerzi, unpublished observations.

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