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ARTICLE *in* BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS · NOVEMBER 1998

Impact Factor: 2.3 · DOI: 10.1006/bbrc.1998.9664

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Binding of *Escherichia coli* Initiation Factor IF2 to 30S Ribosomal Subunits: A Functional Role for the N-Terminus of the Factor

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Received October 8, 1998

In the initiation step of bacterial protein synthesis initiation factor IF2 has to join the 30S ribosomal subunit in order to promote the binding of the fMet-tRNA$_f^{Met}$. In order to identify regions within IF2 which may be involved in the primary ribosome-factor interaction, we have constructed several C-terminal and N-terminal truncated forms of the factor as well as isolated structural domains, and tested them in a 30S ribosomal binding assay *in vitro*. Monoclonal antibodies with epitopes located within the two N-terminal domains of IF2 were used in these experiments. Hitherto, no function has been allocated to the N-terminal region of IF2. Here we show that a mutant consisting of the two N-terminal domains has intrinsic affinity to the ribosomal subunit. Furthermore, a deletion mutant of IF2 which is lacking the two N-terminal domains shows negligible affinity. Moreover mAb with epitopes located within domain II strongly inhibits the binding capacity of IF2 to the 30S ribosomal subunit, whereas mAb with epitopes mapped within domain I do not affect the binding of the factor. The C-terminal domain of IF2 shows no affinity for the small ribosomal subunit. In addition, mutants with C-terminal deletions are not significantly affected in this interaction. Therefore, we conclude that the N-terminus of IF2 has affinity *per se* to bind the ribosomal subunit, with domain II being directly involved in the interaction.

The rate of initiation of protein biosynthesis in *E.coli* is modulated by three initiation factors: IF1, IF2 and IF3 (for reviews see 1, 2). IF2 is the largest of the factors and is expressed in three different forms *in vivo* differing in the N-termini: IF2α (97.3 kDa), IF2β (79.7 kDa) and IF2γ (78.8 kDa) (3).[2]

In the initiation complex formation IF2 interacts with the fMet-tRNA$_f^{Met}$, GTP, IF1, IF3 and both 30S and 50S ribosomal subunits. Through these interactions, IF2 promotes the binding of the initiator tRNA to the 30S ribosomal subunit and catalyses the hydrolysis of GTP following 70S initiation complex formation.

The molecular mechanism by which IF2 interacts with the 30S ribosomal subunit and how this is affected by the rest of the translation machinery, has been studied for more than two decades. Crosslinking experiments have revealed that IF2 covers a large part of the head and caps the lateral protrusion of the subunit (5, 6, 7). However, much less is known concerning the regions or domains within the factor involved in this macromolecular recognition. Unfortunately, neither NMR spectroscopy nor X-ray crystallography have been successfully applied to this molecule. The use of different mutants and truncated forms for the factor in different experiments *in vivo* and *in vitro* has tried to circumvent this problem. It has been shown that the major active centers of IF2 reside in the C-terminal two-thirds of the protein leaving an unknown role for the N-terminus of IF2 (8, 9, 10).

In the present work we have studied the ability of different truncated forms and isolated structural domains of IF2, to bind the 30S ribosomal subunit in an assay *in vitro* in the absence of mRNA, initiator tRNA, IF1 and IF3. Furthermore, we have included monoclonal antibodies with defined epitopes within the N-terminus of IF2 in the assay, aiming at elucidating a refined model of the interaction between IF2 and the 30S ribosomal subunit. The different truncated forms

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Abbreviations: BSA, bovine serum albumin; DTT, dithiothreitol; mAb, monoclonal antibody; PMSF, phenylmethylsulfonyl fluoride.

[2] A new nomenclature for translation factors has been proposed by IUBMB (4). According to this, IF2α and IF2β are named IF2-1 and IF2-2 respectively and IF2γ would be IF2-3.

0006-291X/98 $25.00

of IF2 studied here and the discussion of the results obtained will be related to our six-domain structural model for IF2 described in 11-13.

MATERIALS AND METHODS

Chemicals. All the chemicals used were of analytical grade from Merck or Sigma unless otherwise indicated.

Buffers. Digest-buffer: 200 mM Tris HCl (pH 8.5). *Buffer A:* 50 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 1 mM DTT, 0.1 mM PMSF. *Ribosome binding buffer:* 10 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 100 mM NH_4Cl, 1 mM DTT, 2 mM GTP.

IF2α and IF2β. Both native forms of the initiation factor were overexpressed and purified essentially as described by Mortensen *et al.* (14).

Construction, overexpression, and purification of C-terminal deletion mutants. A battery of 3′ deletion mutants of *infB* were prepared as previously described (13). The DNA fragments containing the *infB*-3′ deletion mutants were excised with *Eco*RI and *Hin*dIII restriction enzymes and ligated into the runaway expression vector pCP40. The new plasmid construction was transformed into the *E. coli* strain UT5600[pCI857] (15). Cell growth, induction of overexpression, cell lysis and column chromatography was performed essentially as described by Mortensen *et al.* (14). The identity and purity of the purified truncated forms was determined by SDS-PAGE followed by Coomassie brilliant blue R staining and western-immunoblotting.

Construction, overexpression, and purification of the N-terminal deletion mutant. A 5′ deletion mutant *infB* Δ1-867 was created by oligonucleotide site-directed mutagenesis by the "gapped duplex" method (16). This mutant gene expresses the N-terminal truncated IF2α Δ1-289, which is identical to the proteolytic fragment obtained when IF2 is cleaved by the outer membrane protease OmpT (17). Protein technology was accomplished essentially as described by Mortensen *et al.* (14), except for the substitution of the cation exchange chromatography by gel filtration through a C16/100 Pharmacia column packed with AcA44 (BioSepra Inc. France).

Proteolysis of IF2 and purification of the C-terminal trypsin-resistant fragment. Fifty mg of pure IF2α were incubated in 5 ml of digest buffer containing 0.1% trypsin (L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated) for 24 h at 4°C. The reaction was stopped by the addition of 0.1% soybean trypsin inhibitor and PMSF to a final concentration of 0.1 M, incubating for 30 min at 20°C. The mixture was then loaded onto a 1 ml Resource Q column (Amersham Pharmacia) equilibrated with buffer A. Proteins were eluted with a linear gradient (0-500 mM) of NaCl in buffer A. Fractions containing pure fragment, as determined by SDS-PAGE, were subsequently dialyzed against buffer A + 100 mM NaCl and 50% glycerol, and stored at -20°C. Furthermore, the purified fragment was characterized by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (18), and the N-terminal amino acid sequence determined by analysis by standard Edman degradation essentially as described in (3). The C-terminal trypsin resistant fragment is named as domain VI.

Monoclonal antibodies against IF2α. Monoclonal antibodies against IF2 were prepared according to Mortensen *et al.* (13).

Preparation of 30S ribosomal subunits. Ribosomes were prepared from MRE600 *E.coli* cells essentially as described in (19) and 30S ribosomal subunits isolated by zonal centrifugation essentially as described in (20). Salt-washed 70S ribosomes (8000 OD_{260}) were loaded on a 1000 ml 8.2-37% hyperbolic zonal sucrose gradient and ultracentrifuged in a Beckman Ti-15 rotor for 27000 rpm/19 h at 4°C. After separation of the ribosomal particles by ultracentrifugation the homogeneity of the subunits was tested by ultracentrifugation through an analytical sucrose gradient and by SDS-PAGE.

30S binding assay. The 30S ribosomal subunits (100 pmol, reactivated at 37°C for 5 min) were titrated with different truncated forms of IF2 (10-200 pmol) incubating for 5 min at 18°C in a final volume of 50 μl ribosome binding buffer. No significance differences was found at 30°C and 37°C. The reaction was stopped by loading it on top of an ice-cold 50 μl sucrose cushion (10% in binding buffer) keeping the temperature constant at 4°C. Samples were subsequently ultracentrifuged for 13 min/4°C at 75000 rpm in a Beckman TL-100 Ultracentrifuge (rotor TLA-100.3). Samples of 50 μl from the upper supernatant, as well as the ribosomal pellets were subsequently analyzed by SDS-PAGE followed by Coomassie brilliant blue G-250 staining and western-immunoblotting (21). Protein quantification was performed as described by Neumann, U. (21) and by densitometric scanning of the gels after staining.

30S binding assay including mAb. IF2α and the C-terminal mutant consisting in the two N-terminal domains of IF2 were preincubated for 30 min at 37°C with 3.5 molar excess of specific mAb before incubation with the ribosomal subunits. After ultracentrifugation, the pellets were analyzed as described above.

RESULTS

Expression and purification of truncated forms of IF2. *E. coli* strain UT5600[pcI857] was transformed with the different deletion mutants of *infB* cloned into pCP40. This cell strain is lacking the outer membrane protease OmpT, thus avoiding the cleavage of IF2 during the purification process mainly at position K_{289}-R_{290} as earlier reported (8). For all the overexpressed mutants, induction conditions of 1h/42°C seemed to be optimal to obtain the highest yield of expressed truncated protein. Under these conditions, the amount of recombinant protein expressed in the cell was determined to be between 30% and 50% of the total soluble fraction, depending on the mutant. It could be observed by SDS-PAGE that both, the α and the β/γ forms, were expressed for all mutants studied with the obvious exception of the OmpT fragment. However, only the larger form were purified . A schematic representation of the expressed truncated forms of IF2 is shown in Fig. 1. The final yield of protein varied between 20-170 mg/10 g cells (wet weight) with a purity over 95% (Fig. 2).

Purification and characterization of domain VI. The IF2-domain VI was prepared by trypsin treatment of IF2α followed by anion exchange chromatography to remove smaller fragments and traces of trypsin and trypsin inhibitor. The C-terminal trypsin resistant fragment eluted in the range 120-150 mM NaCl and by this method, 10 mg of very pure (>95%) IF2-domain VI were obtained. The N-terminus of the domain was determined to be K_{673}LENMFAN and mass spectrometry revealed an intact IF2 on its C-terminus although the C-terminal tripeptide T-I-A was not identified. The purified domain VI is seen in Fig. 2, lane 5.

Isolation of 30S ribosomal subunits. A final yield of 1400 OD_{260} of homogenous 30S ribosomal subunits was obtained as determined by analytical sucrose density gradient ultracentrifugation. Moreover SDS-

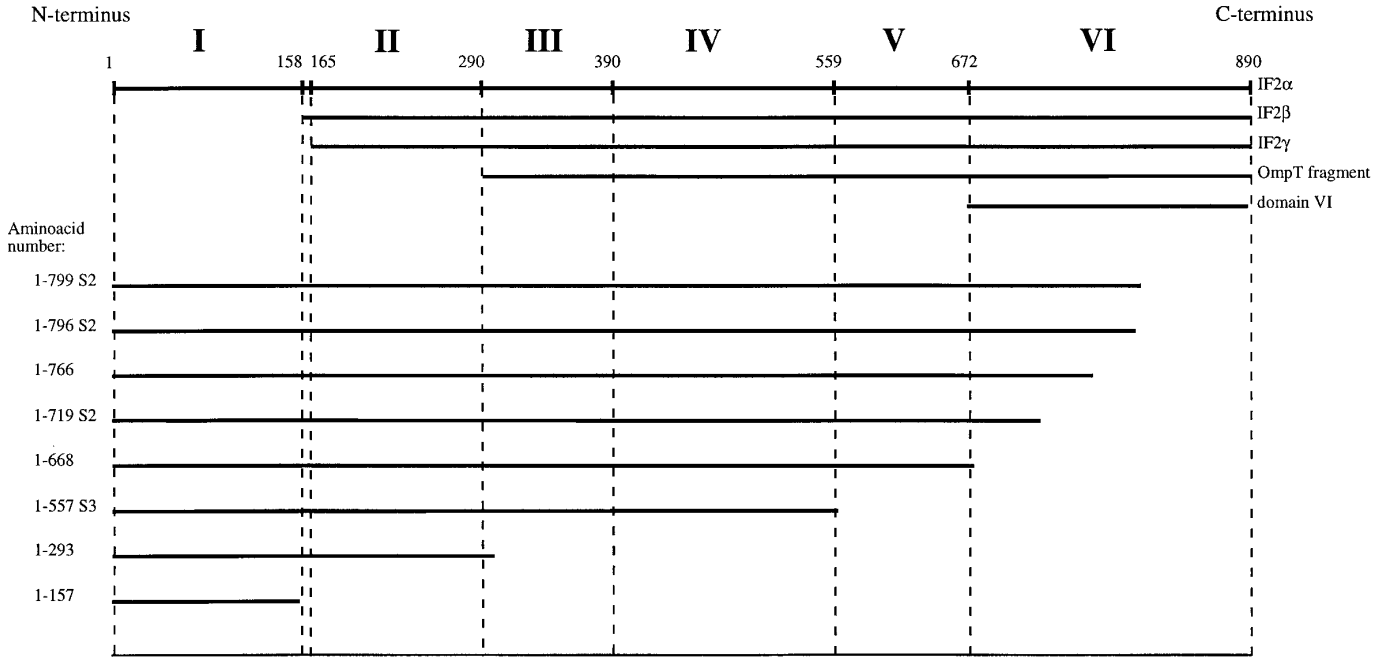

FIG. 1. Schematic representation of the three naturally existing forms of IF2: IF2α, β and γ, as well as the engineered OmpT fragment, the trypsin resistant fragment domain VI, and different C-terminal deletion mutants. The amino acid numbers indicated in the column to the left specify the sizes of the mutants when initiated at the AUG start codon of IF2α. Alternative stop codons of the mutants are designated S2 and S3. The domains of IF2 are numbered I-VI according to the six domain model proposed (11).

PAGE showed no presence of ribosomal proteins from the 50S subunit and the 30S subunit preparation was devoid of detectable IF2 as determined by western-immunoblotting.

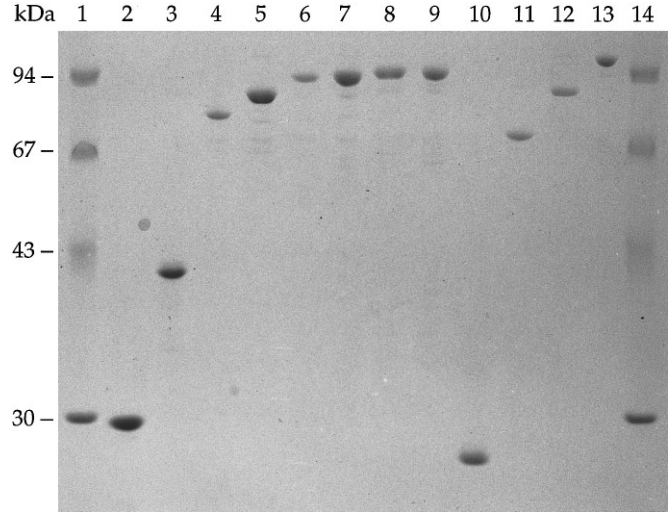

FIG. 2. SDS-PAGE and Coomassie brilliant blue G staining of purified native forms and different truncated forms of IF2 included in the 30S ribosomal assay. Lanes 1 and 14: Molecular weight markers; lane 2: IF2α Δ158-890; lane 3: IF2α Δ294-890; lane 4: IF2α Δ558-890; lane 5: IF2α Δ669-890; lane 6: IF2α Δ720-890; lane 7: IF2α Δ767-890; lane 8: IF2α Δ797-890; lane 9: IF2α Δ800-890; lane 10: domain VI; lane 11: OmpT fragment; lane 12: IF2β; lane 13: IF2α. Samples contain 2-5 μg of protein.

Interaction of IF2 with 30S ribosomal subunits. In order to define the optimal conditions for IF2 interaction with 30S, the ribosomal subunits were titrated with increasing amount of the recombinant IF2 proteins. The protein-ribosome complex was separated from unbound initiation factor by loading the incubated reaction on top of an ice-cold 10% sucrose cushion followed by ultracentrifugation. Fast ultracentrifugation and small sample volume made it possible to pellet the ribosome-protein complex in 13 min avoiding equilibrium problems.

Based on the titration results (data not shown) and the kinetic parameters of Pon *et al.* (22), we performed a series of experiments with equimolar amount (100 pmol) of protein factor and ribosomal subunit, giving final experimental concentrations similar to what has been proposed as conditions *in vivo* (1). Results from these experiments are shown in Fig. 3 and summarized in Table 1.

A mutant consisting in the two N-terminal domains of IF2α could be bound to the ribosomes under different experimental conditions, whereas no detectable affinity of the N-terminal domain of IF2α, domain I, to the 30S ribosomal subunit was found. These results are unexpected since no direct interactions have been reported between the ribosome and the N-terminus of IF2, which moreover has been considered to be functionally dispensable in protein synthesis (8). In addition, the OmpT fragment, lacking the two N-terminal

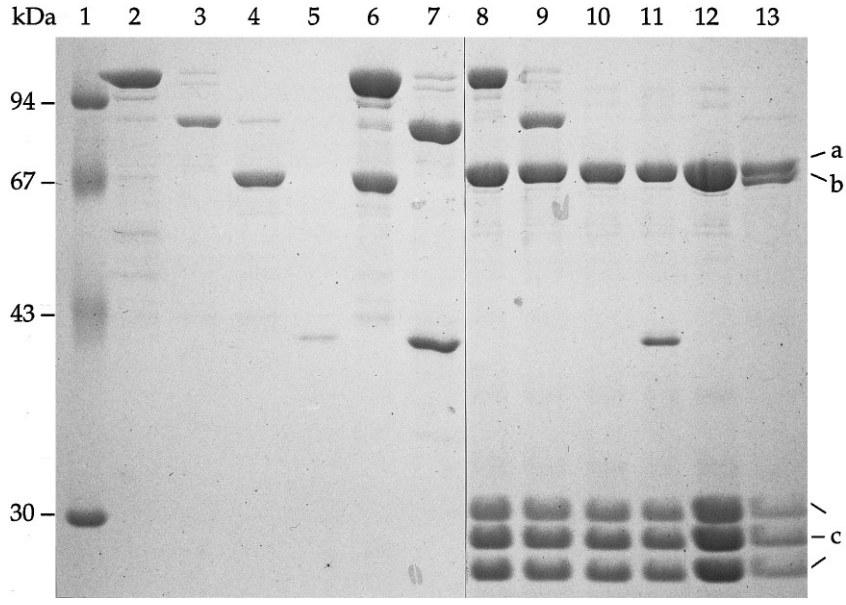

FIG. 3. SDS-PAGE and Coomassie brilliant blue G staining of samples after ribosomal assay. The different recombinant IF2-proteins were incubated with 30S ribosomal subunits in an equimolar amount (2 μM) to be subsequently ultracentrifuged. Supernatant samples as well as ribosomal pellets were then analyzed. When no ribosomes were included in the assay, no truncated proteins were found in the pellet (samples not shown). Lane 1: Molecular weight marker. Lanes 2-5 upper 50 μl supernatants: lane 2: IF2α; lane 3: IF2β; lane 4: OmpT fragment; lane 5: IF2α Δ294-890; lane 6 marker: IF2α and the OmpT fragment, 100 pmol each; lane 7 marker: IF2β and IF2α Δ294-890, 100 pmoles each. Lanes 8-11 pellets: lane 8: IF2α; lane 9: IF2β; lane 10: OmpT fragment; lane 11: IF2α Δ294-890; lane 12: marker, 100 pmol 30S ribosomal subunits; lane 13: markers, **a** and **c** S1 and rest of proteins from 30S ribosomal subunits, respectively, **b** OmpT fragment.

domains of IF2α, showed a negligible affinity for the 30S ribosomal subunit. No interaction between the isolated domain VI of IF2 and the 30S ribosomal subunits could be achieved. Besides this, progressively deleted C-terminal mutant within domain VI and moreover mutants lacking domains VI and V+VI could bind the ribosomal subunit with no significant reduction in their affinity as compared to the wild-type forms for the factor IF2α and IF2β/γ (Table 1).

TABLE 1

Binding Efficiency to the 30S Ribosomal Subunits of the Different Native and Truncated Forms of IF2

Protein studied	30S binding
IF2α	+++
IF2β	+++
domain VI	−−−
IF2α Δ800-890	+++
IF2α Δ797-890	+++
IF2α Δ767-890	+++
IF2α Δ720-890	+++
IF2α Δ669-890	+++
IF2α Δ558-890	+++
IF2α Δ294-890	+++
IF2α Δ158-890	−−−
OmpT fragment	−−−
BSA, trypsin inhibitor ovalbumin, lysozyme	−−−

Note. Values represented come from the analysis of the protein bound to the ribosomes in the pellet as well as the non-bound fraction present in the supernatant, subsequently being compared with experiments in the absence of ribosomal subunits. +++ 80–100%, −−− 0–10%.

Inhibitory effect of monoclonal antibodies. Five different specific mAb mapped within the N-terminal two domains of IF2α were included in the binding assays in order to test which regions within the N-terminus of IF2 could be responsible for the binding to the 30S ribosomes (Table 2) presuming that the binding of an antibody to a protein antigen, at or near a region involved in the interaction by the native or truncated factor with the ribosomal subunit, would disrupt this activity.

TABLE 2

mAb Included in the 30S-IF2 *in Vitro* Assays with Description of Epitopes

mAb	Domain	Amino acid residue	Amino acid sequence
1	I	24-37	FADAGIRKSADDSV
2	I	108-136	AQREAEEQARREAEESAKREA QQKAEREA
3	II	165-179	MTKNAQAEKARREQE
4	II	180-188	AAELKRKAE
5	II	286-293	KGGKRKGS

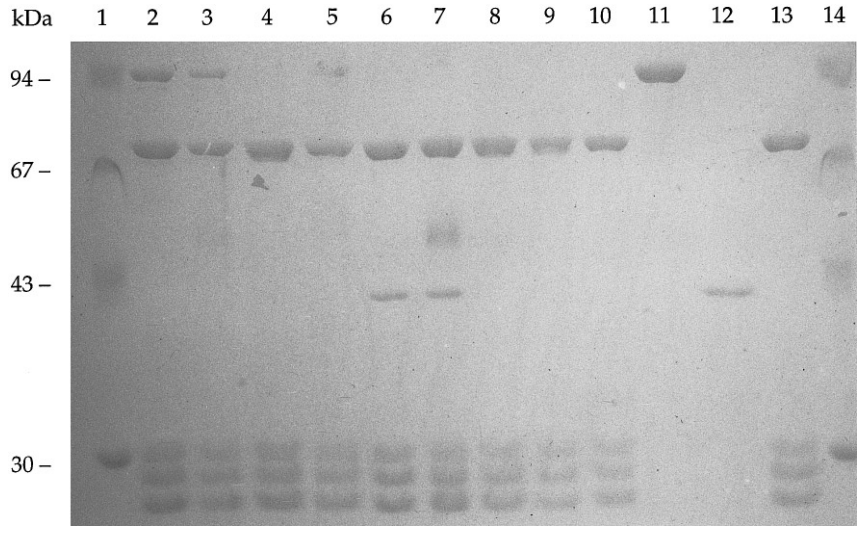

FIG. 4. SDS-PAGE and Coomassie brilliant blue G staining of samples after ribosomal assay including mAb. IF2α and IF2α Δ294-890 were incubated for 30 min at 37°C with 3.5 molar excess of specific antibodies previously to the incubation with the ribosomal subunits. After ultracentrifugation, the pellets were analyzed as shown. Lanes 1 and 14 molecular weight markers. Lanes 11-13: 100 pmol IF2α, 100 pmol IF2α Δ294-890 and 50 pmol of 30S subunits respectively. Lane 2: IF2α + no mAb; lane 3: IF2α + mAb 1; lane 4: IF2α + mAb 4; lane 5: IF2α + mAb 5; lane 6: IF2$\alpha\Delta$294-890 + no mAb; lane 7: IF2α Δ294-890 + mAb 1; lane 8: IF2α Δ294-890 + mAb 4; lane 9: IF2α Δ294-890 + mAb 5; lane 10: experiment including only ribosomal subunits.

The results exemplified in Fig. 4 showed that the mAb nº 1 and 2, both with epitopes located within domain I, did not significantly affect the binding of either IF2α or deletion mutant IF2α Δ294-890 to the 30S ribosomal subunit whereas mAb 3, 4 and 5 with epitopes mapped within domain II of IF2α seem to affect the binding of both, to the limit of total inhibition of the binding to the ribosomal subunit. The results of these experiments are summarized in Table 3.

The results obtained in the ribosomal binding assay including mAb are in agreement with the results previously shown in which the isolated domain I had no affinity for the 30S ribosomal subunit supporting the hypothesis that domain II of IF2α may be important for the binding of the factor to the 30S ribosomal subunit.

TABLE 3

Effect of a Specific mAb Bound to IF2α or the Mutant IF2α Δ294–290 on Its Binding to the 30S Ribosomal Subunit

mAb with epitope at amino acid residue	IF2α	IF2α Δ294-890
24-37	++	+++
108-136	++	+++
165-179	++	++
180-188	−−−	−−−
286-293	−−−	−−−

Note. Percentage of protein bound compared to an experiment with no antibodies: +++ 80–100%, ++ 40–80%, −−− 0–10%.

DISCUSSION

The work reported here presents new information concerning the interaction between IF2 and the 30S ribosomal subunit. The C-terminal domain of IF2 is believed to be crucial for promoting the binding of the initiator tRNA to the 30S ribosomal complex (10, 23, 24). However, we have found no evidence of a direct interaction between the isolated domain VI and the 30S ribosomal subunit. This is in agreement with earlier results obtained by Gualerzi *et al.* (10) who showed that a C-terminal trypsin resistant fragment from *Bacillus stearothermophilus* lacked completely the affinity for the 30S ribosomal subunit. In addition, a genetically engineered G-domain, which would correspond to domains III+IV+V of *E. coli* IF2, retained the capacity for binding to the 50S ribosomal subunit, whereas the binding to the 30S subunit was very weak. Since we have shown that a C-terminal truncated form of IF2 lacking domains V+VI was able to bind to the 30S ribosomal subunit and that IF2-domain VI does not bind to the 30S ribosomal subunit, it is tempting to consider the possibility of having domains involved in the interaction with the subunit towards the N-terminus of IF2.

Hitherto, no functions have been allocated to the N-terminus of IF2. However, we have isolated a mutant consisting of domains I+II which binds to the 30S ribosomal subunit. The isolated N-terminal domain of IF2α (domain I) does not bind to the ribosomal subunit and mAb with epitopes located within domain I, once bound to IF2, do not affect the binding capacity of the

native factor. These results combined with non-binding of the OmpT fragment and the inhibitory effect of mAb with epitopes within domain II lead us to conclude that this domain II, the N-terminal domain of IF2β is directly involved in the binding of IF2 to the 30S ribosomal subunit.

For at least two reasons, functional importance of the N-terminus of IF2 has been neglected until now. First, in our earlier experiments *in vitro* with mutants lacking N-terminal domains similar specific activities were found for IF2α and the OmpT fragment in assays for binding of initiator tRNA to the 70S ribosome complex and for GTP hydrolysis, indicating that the catalytic centers of IF2 were located within the C-terminal two thirds of the molecule (8). However, recalling these results it is observed that the activity of the truncated form as compared to IF2α was affected by the presence of IF1 and IF3. The fMet-tRNA$_f^{Met}$ binding is a complex process which involves both ribosomal subunits, IF1, IF3, mRNA and aminoacylated and formylated initiator tRNA. IF1 and IF3 are known to stimulate the binding of IF2 to the 30S ribosomal subunit, and moreover to increase the binding efficiency of the fMet-tRNA$_f^{Met}$ in the presence of IF2 (2, 25, 26). The experiments described in this paper do not include IF1 and IF3, but provides information about intrinsic affinity of IF2 domains to the 30S ribosomal subunit. We can therefore not exclude that inclusion of IF1 and/or IF3 would increase the affinity of other parts of IF2 to the 30S subunit. Second, by comparing all known IF2 amino acid sequences from different bacterial species, a common characteristic feature has appeared: the N-terminal part is highly variable whereas the C-terminal two thirds of the factor seem to be evolutionarily conserved. This has lead to propose that the N-terminus of IF2 could be functionally dispensable in protein synthesis. However, we have recently shown that, despite inter species variations, IF2 is an extremely conserved protein in *E. coli*, especially on its N-terminus. We conclude that the N-terminus of IF2 must be important for the cell in its natural habitat since the three initiation sites for the natural forms have been conserved in a large number of clinical and environmental isolates (12). Indeed, although *E. coli* has been shown to be able to grow at laboratory conditions when lacking IF2α or IF2β, it was found that maximal growth required the presence of both forms (27).

In this article we have proposed a functional role for the N-terminus of IF2: a direct interaction of the domain II with the 30S ribosomal subunit. Possible interactions between the truncated forms of IF2 and the rest of the translation machinery, in particular IF1 and IF3, are presently being studied aimed at refining the functional model for IF2.

ACKNOWLEDGMENTS

We thank Hans Frede Hansen for MALDI technical assistant. This work was supported by grants from the Commission of the European Communities (Contract no CHRX-CT-94-0529), Familien Hede Nielsens Fund and the Biotechnology Programme of the Danish Natural Sciences Research Council (28807-9502036, 9602401) to HUSP. JMPM received a visiting fellowship from the Danish Forskerakademi.

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