*Articles*

# Structure–Function Analysis of *Escherichia coli* Translation Initiation Factor IF3: Tyrosine 107 and Lysine 110 Are Required for Ribosome Binding†

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ABSTRACT: Translation initiation factor IF3 is required for peptide chain initiation in *Escherichia coli*. IF3 binds directly to 30S ribosomal subunits ensuring a constant supply of free 30S subunits for initiation complex formation, participates in the kinetic selection of the correct initiator region of mRNA, and destabilizes initiation complexes containing noninitiator tRNAs. The roles that tyrosine 107 and lysine 110 play in IF3 function were examined by site-directed mutagenesis. Tyrosine 107 was changed to either phenylalanine (Y107F) or leucine (Y107L), and lysine 110 was converted to either arginine (K110R) or leucine (K110L). These single amino acid changes resulted in a reduced affinity of IF3 for 30S subunits. Association equilibrium constants ($M^{-1}$) for 30S subunit binding were as follows: wild-type, $7.8 \times 10^7$; Y107F, $4.1 \times 10^7$; Y107L, $1 \times 10^7$; K110R, $5.1 \times 10^6$; K110L, $<1 \times 10^2$. The mutant IF3s were similarly impaired in their abilities to specifically select initiation complexes containing $tRNA^{fMet}$. Toeprint analysis indicated that 5-fold more Y107L or K110R protein was required for proper initiator tRNA selection. K110L protein was unable to mediate this selection even at concentrations up to 10-fold higher than wild type. The results indicate that tyrosine 107 and lysine 110 are critical components of the ribosome binding domain of IF3 and, furthermore, that dissociation of complexes containing noninitiator tRNAs requires prior binding of IF3 to the ribosomes.

Initiation of protein biosynthesis in *Escherichia coli* requires the specific interaction of a 30S ribosomal subunit, initiator fMet-tRNA, and the initiator region of mRNA. Translation initiation factor IF3[1] plays a crucial role in this process by ensuring that a pool of free 30S ribosomal subunits is available and by mediating the functional interaction between mRNA and the 30S subunit (Maitra et al., 1982; Gualerzi & Pon, 1990). More recently, participation of IF3 in the selection of the correct mRNA initiator region and destabilization of initiation complexes containing noninitiator tRNAs has been demonstrated (Gualerzi & Pon, 1990; Hartz et al., 1989; 1990).

IF3 is a basic protein of 180 amino acids with an apparent molecular weight of 20 600 (Brauer & Wittman-Liebold, 1977; Sacerdot et al., 1982). The 30S subunit binding site for IF3 has been extensively characterized by a variety of approaches. The 30S subunit proteins S7, S11, S12, S18, and S21 have been consistently cross-linked to bound IF3 by chemical and photochemical means (Heimark et al., 1976; MacKeen et al., 1980; Cooperman et al., 1981; Boileau et al., 1983). Several regions of 16S rRNA have also been implicated in IF3 binding, including nucleotides within the central domain (690–850) and others at the 3′ end (1506–1529) (Ehresmann et al., 1986; Muralikrishna & Wickstrom, 1989). In addition, mutation of the conserved nucleotides at positions 791 and 792 in 16S rRNA resulted in loss of IF3 binding activity (Tapprich et al., 1989; Santer et al., 1990).

Significantly less is known regarding the structural elements of IF3 which are required for activity. Gualerzi and co-workers have probed the structure–function relationships of IF3 by chemical modification and have identified several amino acid residues which may be essential for function (Bruhns & Gualerzi, 1980; Ohsawa & Gualerzi, 1981; Lammi et al., 1982). In particular, modification of lysine 112[2] resulted in complete loss of activity (Ohsawa & Gualerzi, 1981) and iodination of tyrosine 109[2] was accompanied by a loss of 30S subunit binding capacity (Bruhns & Gualerzi, 1980). Chemical modification studies suffer from the inability to target a single amino acid residue and difficulty in distinguishing between a direct effect at the modification site or a more general outcome due to the chemical reaction itself.

Site-directed mutagenesis provides the opportunity to probe structure–function relationships of IF3 with greater specificity. In the present work, several amino acid replacements were engineered into IF3 at residues 107 and 110[2] in order to clarify their roles in IF3 function. Mutant IF3 proteins were purified and assayed for their ability to bind to 30S subunits and proofread the fMet–tRNA–mRNA interaction. The results

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[1] Abbreviations: IF3, initiation factor 3; IPTG, isopropyl β-D-thiogalactopyranoside; X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; FITC, fluorescein isothiocyanate.

[2] The numbering of amino acid residues in IF3 prior to the sequencing of its gene in 1983 (Sacerdot et al., 1983) was inaccurate due to some protein sequencing errors (Brauer & Wittmann-Liebold, 1977). Thus, lysine 112 and tyrosine 109 are more correctly numbered as lysine 110 and tyrosine 107. They will be referred to as such in the remainder of the paper.

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indicate that Y107 and K110 comprise a portion of the 30S subunit binding site of IF3.

## EXPERIMENTAL PROCEDURES

*Chemicals and Enzymes.* Acrylamide (electrophoresis grade), Trizma base, α-D-(+)-glucose, poly(ethylene glycol) (6000), ampicillin, glycine, and Hepes were obtained from Sigma Chemical Co. Agarose (electrophoresis grade), IPTG, ammonium persulfate, X-gal, sucrose (Ultrapure), and phenol (redistilled, nucleic acid grade) were from Bethesda Research Laboratories. [$^{14}$C]Formaldehyde (56 mCi/mmol) was obtained from ICN Biochemicals, sodium cyanoborohydride was purchased from Aldrich Chemical Co., and fluorescein isothiocyanate (isomer I) was from Molecular Probes, Inc.

All restriction endonucleases and T4 DNA ligase were from Bethesda Research Laboratories, and lysozyme was obtained from Sigma Chemical Co.

All other chemicals used were from VWR and were reagent grade unless otherwise specified.

*Plasmid and Phage Vectors Containing infC.* The construction of a plasmid containing *infC*, the gene for IF3 (designated pAP1), was described previously (Pramanik & Schwartz, 1984). A derivative of this plasmid capable of driving large-scale overexpression of IF3 (designated pDD1; see below) has also been described (De Bellis & Schwartz, 1990). For the site-directed mutagenesis experiments described below, a 1109-bp *Hpa*I fragment from pAP1 containing the *infC* gene was ligated into *Sma*I-digested M13mp19 dsDNA.

*Site-Specific Oligonucleotide-Directed Mutagenesis.* Mutagenesis was performed by the method of Kunkel (Kunkel, 1985; Kunkel et al., 1987) using the Bio-Rad Muta-Gene M13 in vitro mutagenesis kit. Authenticity of the mutant phages were confirmed by sequencing of single-stranded phage DNA (Sanger et al., 1977).

To facilitate mutagenesis and large-scale production of IF3, *E. coli infC* was cloned in pUC19 under the control of the *lac* promoter. The details for construction of the *infC* expression vector and introduction of point mutations in the cloned gene are presented in Figure 1. A 1048-bp *Dra*I–*Hpa*I fragment was cloned into the *Sma*I site of pUC19 in two steps to yield the expression plasmid pDD1. Previous studies had shown that the major promoter for expression of *infC* is $P_{12}$ (Pramanik et al., 1986). There is a *Dra*I recognition site in the −10 region of this promoter, and therefore, digestion with this enzyme followed by cloning into the polylinker of pUC19, results in *infC* under the inducible control of the *lac* promoter. Overproduction of IF3 by induction of *infC* expression in cells transformed with pDD1 by IPTG addition has been previously demonstrated (De Bellis & Schwartz, 1990).

*Bacterial Growth. E. coli* strains JM107 or DH5α were grown to mid-log phase in L broth and transformed with the appropriate plasmids as described previously (Elseviers et al., 1982). Transformants containing inserts were selected by plating on L broth-containing agar plates in the presence of 100 μg/mL ampicillin, 33 μM IPTG, and 1 mg of X-gal. After overnight growth at 37 °C, single white colonies were purified by streaking onto fresh ampicillin plates and incubated at 37 °C overnight. Single colonies were grown in 5-mL overnight cultures of L broth/ampicillin, and plasmid DNA was isolated by the alkaline extraction procedure of Morelle (1989). Desired plasmid constructs were identified initially by restriction analysis and confirmed by sequencing of the plasmid DNA as described by Chen and Seeburg (1985).

*Overproduction and Purification of Wild-Type and Mutant IF3. E. coli* JM107 or DH5α transformed with the appropriate plasmids were grown in media containing (per liter) 17 mmol of $KH_2PO_4$, 72 mmol of $K_2HPO_4$, 12 g of tryptone, 24 g of yeast extract, 4 mL of glycerol, and 100 mg of ampicillin. Cultures were incubated at 37 °C until the late-log phase of growth (2–4 h). Cultures were then adjusted to 4 mM with respect to IPTG, and incubation was continued for an additional 3 h. Cells were harvested by centrifugation, washed, and resuspended in 10 mM Tris–HCl, pH 7.4 and 10 mM $MgCl_2$. The typical cell yield from 5 L of culture was 18 g of wet weight.

IF3 was purified by a modification of the procedure of Hershey et al. (1977). A crude ribosomal fraction was obtained by grinding the cells at 4 °C with an equal weight of alumina and sequential centrifugations at 10000*g* for 30 min to remove cell debris, at 30000*g* for 45–60 min, and overnight at 35000 rpm in a Beckman Ti-45 rotor at 4 °C to pellet the ribosomes. This pellet was resuspended in TMAI buffer [10 mM Tris–acetate, pH 7.8, 10 mM $Mg(OAc)_2$, 30 mM $NH_4(OAc)$, 7 mM β-mercaptoethanol, 0.1 mM EDTA], $NH_4Cl$ was added to a final concentration of 1.0 M, the solution was stirred at 4 °C for 3 h, and the salt wash fraction was obtained by centrifugation through a 20% glycerol cushion in 20 mM Tris–HCl, pH 7.4, 1.0 M $NH_4Cl$, 40 mM $Mg(OAc)_2$, and 7 mM β-mercaptoethanol at 35 000 rpm in a Beckman Ti45 rotor for 17 h. A 45–80% ammonium sulfate fraction was obtained from the supernatant; the IF3-containing pellet was resuspended in 10 mM potassium phosphate, pH 7.5, 0.1 mM EDTA, 7 mM β-mercaptoethanol, 5% glycerol, and 500 mM KCl [buffer A(500)] and dialyzed overnight against 2 L of buffer A(500). The retentate was applied to a 1.5 cm × 25 cm phosphocellulose column which had been preequilibrated with buffer A(500). A 350-mL linear gradient of 0.5–1.0 M KCl in the same buffer was applied to the column. IF3 eluted between 680 and 810 mM KCl and $NaDodSO_4$–polyacrylamide gel electrophoretic analysis revealed that the peak fractions contained essentially pure IF3 (>95%). The IF3-containing fractions were pooled and the protein was concentrated by reapplication to, and batch elution from, a phosphocellulose minicolumn (1-mL total bed volume). The standard procedure for purification of IF3 requires two phosphocellulose column chromatography steps followed by concentration on a third phosphocellulose column. The method developed here required only one phosphocellulose chromatography step prior to concentration on a 1-mL phosphocellulose minicolumn. Yields as high as 0.6 mg of pure IF3/g of cells have been obtained with cells harboring the wild-type plasmid pDD1.

Protein concentrations were determined by a modification of the method of Bradford (1976) using a protein assay kit obtained from Pierce Chemical Co.

*Isolation of 70S Ribosomes and 30S Ribosomal Subunits.* "Tight-couple" 70S ribosomes and 30S ribosomal subunits were prepared as previously described (Stahli & Noll, 1977; MacKeen et al., 1980).

*Determination of Association Equilibrium Constants for IF3–30S Subunit Interaction.* The association equilibrium constants for the binding of IF3 to 30S subunits were ascertained using fluorescently labeled IF3 essentially as described (Tapprich et al., 1989). Purified IF3 (0.3–1.1 μg/μL) was fluorescently labeled with fluorescein isothiocyanate (FITC) by incubating 10–50 μL of protein with 2–5 μL of FITC (0.2 mg/mL) in a total volume of 25–50 μL for 30 min at room temperature. Reaction volumes varied according to

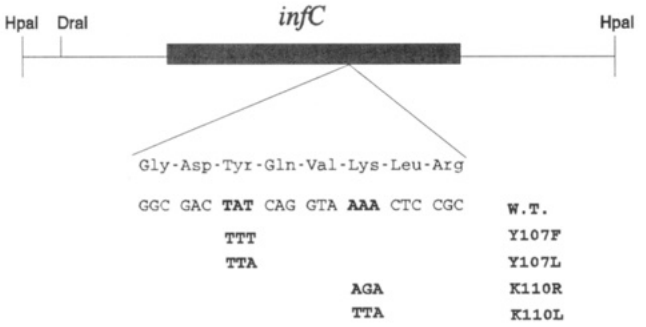


FIGURE 1: Mutations introduced into IF3. A schematic diagram showing *infC* and the restriction sites employed in the cloning described in the text is presented. The IF3 amino acid sequence from positions 105 to 112 and the corresponding nucleotide sequences are shown. The codons targeted for mutagenesis and the sequences of the mutants are given in bold type.

protein concentration and labeling efficiency. Unincorporated FITC was removed by passage over a 1-mL Sephadex G-10 column.

Binding of IF3 to 30S subunits was carried out by titrating increasing amounts of 30S subunits to a fixed amount of FITC-labeled IF3 protein, as follows. Varying amounts of labeled protein were diluted to 100 $\mu$L with FITC dilution buffer (10 mM Tris–HCl, pH 7.8, 50 mM KCl, 2 mM $MgCl_2$) and transferred to a 1-mL fluorescence cuvette. Aliquots of 30S subunits were added to the cuvette, mixed gently by hand, and incubated for 1 min at room temperature. Fluorescence was measured at an excitation wavelength of 490 nm and an emission wavelength of 516 nm. All fluorescence measurements were corrected for the dilution of the sample during the course of the titration.

Association equilibrium constants ($K_a$) were determined from the calculated slopes of Eadie–Hofstee plots (Eadie, 1942) obtained by plotting the change in fluorescence ($\Delta F$) vs $\Delta F/c_f$ ($M^{-1}$), where $c_f$ is the concentration (after correction) of 30S subunits added. $\Delta F$ was calculated from the difference in fluorescence intensity at 516 nm of IF3 before addition of ribosomes ($F_i$) and after each addition of 30S subunits ($F_j$); thus, $\Delta F = F_i - F_j$.

*IF3-Induced Dissociation of 70S Ribosomes.* Ribosomes (720 pmol/mL) were incubated in 50 mM Tris–HCl, pH 7.4, 100 mM $NH_4Cl$, 1 mM DTT, and 5 mM $MgCl_2$ for 5 min at 25 °C in the presence of a 1–10-fold molar excess of IF3. Reaction mixtures were centrifuged at 20 500 rpm through 10–30% sucrose gradients in the above buffer for 17.5 h in a Spinco SW41 rotor. Gradients were then fractionated through a flow cell in a LKB Ultrospec spectrophotometer and the $A_{260}$ was monitored. The extent of dissociation was determined by comparison of the areas under the 30S, 50S, and 70S peaks.

*Extension Inhibition.* Extension inhibition ("toeprint") analysis of $tRNA^{fMet}$–ribosome–mRNA complex formation was performed essentially as described (Hartz et al., 1989) using the RS170 transcript (comprising nucleotides –92 to +107 of bacteriophage T4 gene 32 mRNA) as the template and 32loopD (complementary to +60 to +80 of T4 gene 32 mRNA) as the primer.

## RESULTS

*Mutagenesis of infC and Overexpression of IF3.* Mutants in IF3 with single amino acid changes at position 107 or 110 were produced. Figure 1 shows the amino acid and nucleotide sequence for wild-type IF3 and *infC*, respectively, and the corresponding mutations which have been introduced into *infC*. Tyrosine at position 107 was converted to either phenylalanine (Y107F) or leucine (Y107L), and lysine at position 110 was mutated to either arginine (K110R) or leucine (K110L). The mutations were confirmed by DNA sequencing of both the M13 phage and plasmid clones.

An expression system was developed for overproduction of IF3 (Experimental Procedures) which provides two important advantages relevant to the current studies. First, it allows for the purification of substantial amounts of IF3 for in vitro studies. Second, it circumvents the potential problems that may arise from construction of mutant forms of IF3 which may prove to be lethal to the cell. The induction and purification scheme which has been devised permits growth of transformed *E. coli* to late-log phase under conditions which keep expression from the *lac* promoter repressed. IF3 is synthesized only upon induction with IPTG.

Due to the amplified expression of IF3, a purification scheme which was significantly faster and simpler than the standard procedure of Hershey et al. (1977) could be employed (see Experimental Procedures). Yields of pure IF3 as high as 0.6 mg/g of cells were obtained from both wild-type and mutant transformants. The IF3 isolated in this manner was predominantly the "short" form. As described below, the association equilibrium constant for the 30S subunit–IF3 interaction determined by use of this IF3 preparation was similar to that previously obtained by others.

*Binding of Wild-Type and Mutant IF3 to 30S Ribosomal Subunits.* The role of Y107 and K110 in the binding of IF3 to 30S subunits was studied by monitoring the comigration of wild-type or mutant IF3 with 30S subunits on sucrose density gradients. Saturation binding curves for wild-type, Y107F, and Y107L IF3 indicated that both mutant proteins were impaired in their ability to interact with 30S subunits (data not shown). To further characterize the 30S subunit binding capacities of mutant IF3s, equilibrium binding constants for the IF3–ribosome interaction were determined by fluorescence titration as previously described (Tapprich et al., 1989).

Fixed amounts of fluorescein-labeled IF3 were titrated with increasing quantities of 30S particles (6.7–107 nM), the changes in fluorescence emission were monitored, and binding constants for the interaction were determined as described in Experimental Procedures. The results from a representative experiment are presented in Figure 2. IF3 fluorescence decreased with increasing additions of 30S subunits until a saturation point was reached. Transformation of the data (Figure 2, inset) yields a linear plot from which the association equilibrium constant ($K_a$) for the interaction can be determined. Equilibrium constants for wild-type and mutant IF3s obtained in this manner are presented in Table I. The binding constant for wild-type IF3 was $7.8 \times 10^7$ $M^{-1}$, which is in good agreement with other values reported for this interaction (Box et al., 1981; Weiel & Hershey, 1981). Each of the mutant proteins, however, demonstrated lower $K_a$ values: $4.1 \times 10^7$ and $1.0 \times 10^7$ $M^{-1}$ for Y107F and Y107L, respectively. Mutations of K110 resulted in proteins more severely impaired in 30S subunit binding capacity. Substitution of the lysine with arginine caused a 15-fold reduction in $K_a$ and replacement with leucine in K110L yielded an IF3 with essentially no 30S subunit binding capacity.

Collectively, these experiments provide evidence that Y107 and K110 are essential for maximum efficiency of IF3 binding to 30S subunits and suggest that these residues comprise a portion of the 30S subunit binding domain of IF3.

*Dissociation of 70S Ribosomes by IF3.* IF3 effects the equilibrium between 70S ribosomes and free 30S and 50S subunits, thus maintaining the intracellular pool of free 30S

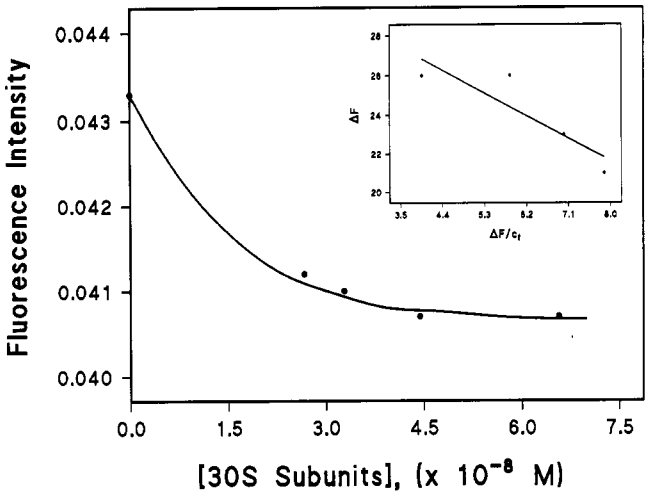


FIGURE 2: Fluorescence intensity of IF3–30S subunit complexes as a function of increasing 30S subunit concentration. Increasing amounts of 30S ribosomal subunits were added to FITC-labeled IF3 (0.7 $\mu$M) in 10 mM Tris–HCl, pH 7.8, 50 mM KCl, and 2 mM $MgCl_2$, and the fluorescence emission at 516 nm (excitation wavelength, 490 nm) was recorded. Inset: Eadie–Hofstee plot of the fluorescence data. $K_a$ is equal to the negative reciprocal of the calculated slope of such a plot.

Table I: Association Equilibrium Constants for Wild-Type and Mutant IF3 Binding to 30S Ribosomal Subunits[a]

| IF-3 | $K_a$, $M^{-1}$ [range][b] | fold reduction[c] |
|---|---|---|
| wild type | $7.8 \times 10^7$ [$(5.7–12.5) \times 10^7$] | |
| 107F | $4.1 \times 10^7$ [$(3.1–5.9) \times 10^7$] | 1.9 |
| 107L | $1.0 \times 10^7$ [$(0.80–1.3) \times 10^7$] | 7.8 |
| 110R | $5.1 \times 10^6$ [$(4.3–6.4) \times 10^6$] | 15 |
| 110L | $<1 \times 10^2$ | $>10^4$ |

[a] Determined with fluorescein-labeled IF3 as described in Experimental Procedures. [b] Range was determined using the standard error of the slope obtained by linear regression analysis. All values were fit within a 95% confidence limit. [c] Relative to wild-type IF3.

subunits required for initiation. Figure 3 illustrates the sucrose density gradient profiles of 70S tight-couple ribosomes in the presence of wild-type or Y107 mutant IF3. In Figure 3A the normal dissociation pattern is evident in the appearance of separate 30S and 50S peaks following incubation of ribosomes with wild-type IF3. The Y107F mutant appears to be equally effective in inducing dissociation; well-defined subunit peaks are clearly discernible at a 5:1 molar ratio of Y107F to ribosomes (Figure 3B). In contrast, the ability of Y107L to mediate dissociation of 70S particles is clearly impaired. Even at a 10-fold molar excess of Y107L over ribosomes, a substantial amount of the ribosomes remain as undissociated 70S couples (Figure 3C). In addition, a distinct 30S subunit peak was not observed, which suggests some alteration in the interaction of Y107L IF3 with the ribosomes.

*Effect of IF3 on Initiator tRNA Selection.* Initiation factors play a role in the specific selection of initiator tRNA during translation initiation (Hartz et al., 1989, 1990). This functional property of IF3 can be evaluated by extension inhibition analysis (or toeprinting) as described initially by Hartz et al. (1988). The assay is based on the inhibition of primer extension by reverse transcriptase on a ribosome-bound mRNA template. Collision of reverse transcriptase with the 30S ribosomal subunit prevents further extension and facilitates determination of the precise position of the bound ribosome. The assay has been employed to investigate the role of IF3 in selection of the correct mRNA–tRNA interaction during initiation complex formation.

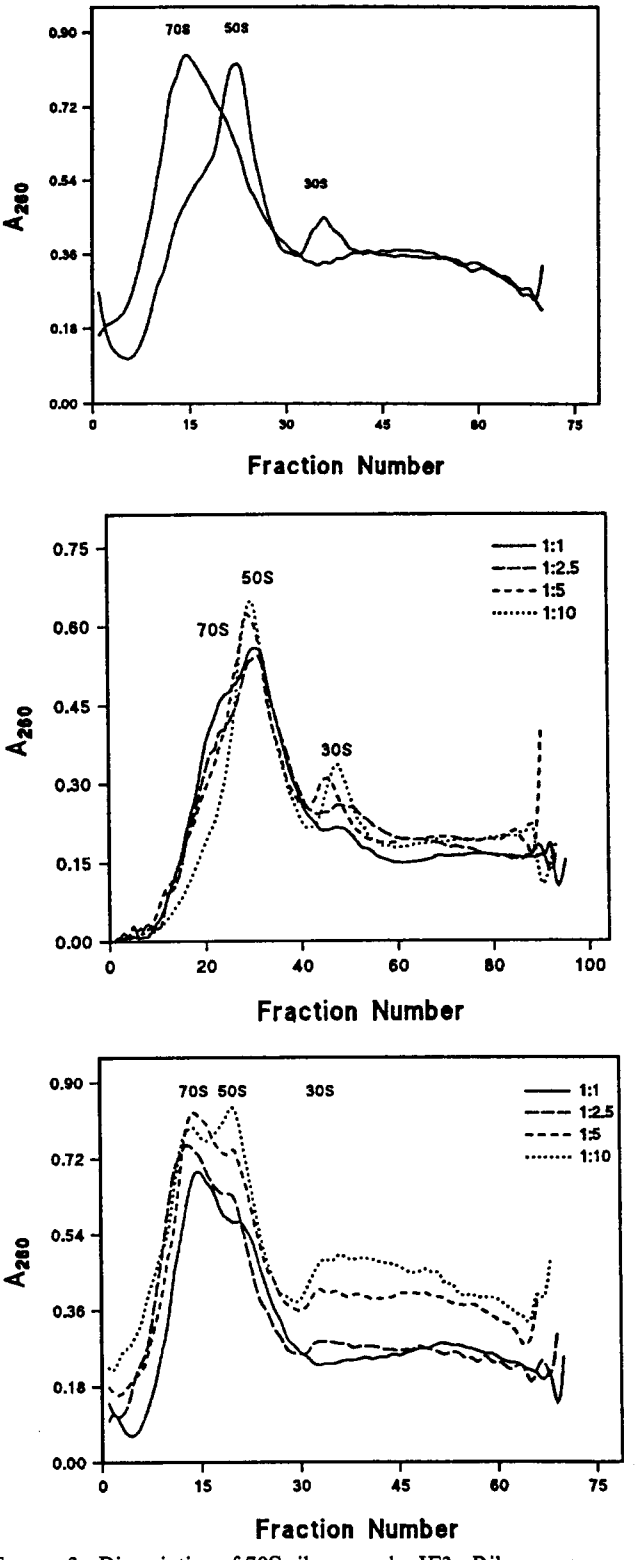


FIGURE 3: Dissociation of 70S ribosomes by IF3. Ribosomes were incubated for 5 min in the presence or absence of IF3 as described in Experimental Procedures. Dissociation into subunits was monitored by sucrose density gradient centrifugation. Migration positions for ribosomes and subunits are indicated in each panel. (A, top) 70S ribosomes in the presence or absence of an 8-fold molar excess of wild-type IF3. (B, middle, and C, bottom) Y107F and Y107L mutant IF3; the molar ratios of ribosomes to IF3 are indicated in the panels.

Initiation complexes containing mRNA, 30S subunits, $tRNA^{fMet}$, and $tRNA^{phe}$ were formed and incubated with reverse transcriptase and a $^{32}P$-labeled primer complementary to a region within the coding sequence of the T4 gene 32 mRNA used as template. After primer extension by reverse transcriptase, the products were separated by electrophoresis

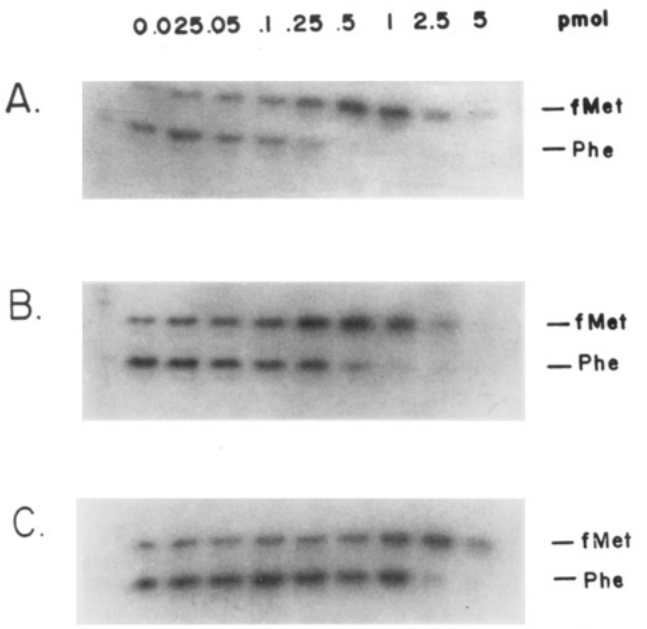


FIGURE 4: Selection of $tRNA^{fMet}$–mRNA–30S subunit ternary complexes by wild-type and Y107 mutant IF3s. Extension inhibition reactions were carried out as described in Experimental Procedures. The mixtures contained 100 nM 30S subunits, 500 nM $tRNA^{fMet}$, 2 $\mu$M $tRNA^{Phe}$, and 6.7 nM mRNA (in vitro-synthesized fragment of bacteriophage T4 gene 32 mRNA from –92 to +107) in a final volume of 10 $\mu$L. The amounts of IF3 added (in pmol) are indicated above each lane. After reverse transcription, reactions were terminated by heating at 95 °C for 2 min and analyzed by electrophoresis on 8% acrylamide gels. Bands corresponding to complexes with $tRNA^{fMet}$ and $tRNA^{Phe}$ are indicated: (A) wild-type IF3; (B) Y107F; (C) Y107L.

on an 8% sequencing gel. Results of a typical toeprinting experiment are presented in Figure 4. In the presence of suboptimal concentrations of IF3, two extension products are observed. The upper (larger size) band corresponds to an extension product inhibited by ribosomes bound correctly at the AUG initiation codon. The lower band is shorter by three nucleotides due to extension inhibition at ribosomes containing $tRNA^{Phe}$ bound at the second codon. This represents incorrect initiation due to improper selection of the initiation codon.

Figure 4A shows the effect of IF3 on initiation codon selection. In the absence of IF3, no discrimination between the correct $tRNA^{fMet}$–AUG complex and the improper $tRNA^{Phe}$–UUU complex occurs. As the concentration of IF3 is increased, $tRNA^{fMet}$ binding is selected at the expense of incorrectly bound $tRNA^{Phe}$. The addition of 0.5 pmol of IF3 to the reaction resulted in exclusive selection of the correct initiation complex. The capacity of Y107F and Y107L to mediate this selection was tested. Y107F was about half as efficient as wild-type IF3 in that 0.5 pmol of added protein resulted in a significant increase in correct vs incorrect complex formation (Figure 4B). Twice as much Y107F (i.e., 1.0 pmol) was required for complete selection of $tRNA^{fMet}$–AUG complex. By contrast, 5-fold higher concentrations (2.5 pmol) of Y107L were required to obtain an equivalent degree of selection.

Similar experiments were conducted with the K110 mutants (Figure 5). K110R had an effect analogous to Y107L; i.e., correct selection required between 2.0 and 5.0 pmol of protein (Figure 5B). K110L was most severely impaired in the "proofreading" function. Essentially no selection of $tRNA^{fMet}$–AUG complex was observed even at 5.0 pmol of added protein. At these high concentrations of protein, the reverse transcriptase reaction itself was inhibited and it was, therefore,

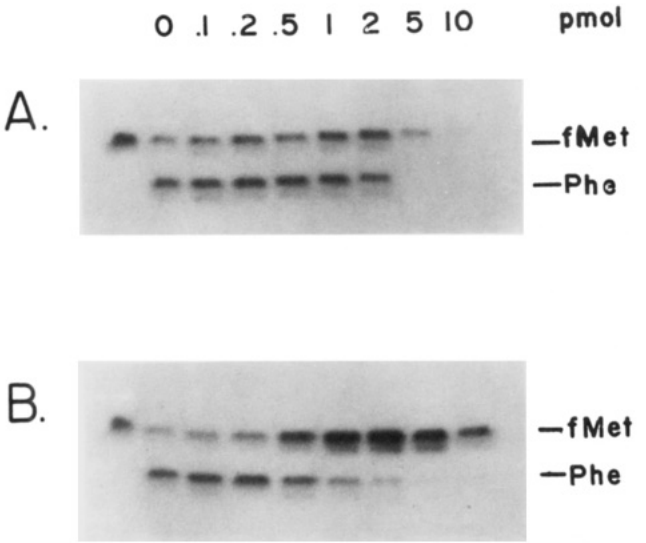


FIGURE 5: Selection of $tRNA^{fMet}$–mRNA–30S subunit ternary complexes by K110 mutant IF3s. Experiments were carried out as described in the legend to Figure 4. The amounts of IF3 added (in pmoles) are indicated above each lane. Unmarked lanes correspond to reactions in the presence of $tRNA^{fMet}$ as the only added tRNA. Bands corresponding to complexes with $tRNA^{fMet}$ and $tRNA^{Phe}$ are indicated: (A) K110L; (B) K110R.

impossible to test IF3 concentrations above 10 pmol. These studies indicate that alterations in the amino acids at position 107 and 110 in IF3 result not only in the loss of ribosome binding ability but also in a loss in the capacity of IF3 to "proofread" the tRNA and anticodon–mRNA interaction as well.

## DISCUSSION

Protein synthesis initiation in *E. coli* is mediated by three initiation factors—IF1, IF2, and IF3. Classically, several activities have been ascribed to IF3. These include a role in maintenance of ribosomal subunits by virtue of dissociation or antiassociation activity (Chaires et al., 1981, 1982; Goss et al., 1982; Kaempfer, 1972; Gottlieb et al., 1975), stimulation of mRNA binding to 30S subunits, and stimulation of initiator tRNA binding to 70S ribosomes (Meier et al., 1973; Vermeer et al., 1973). More recently, a role for IF3 in proofreading the initial tRNA binding step has been described (Hartz et al., 1989, 1990; Gualerzi & Pon, 1990). Given the multiple roles ascribed to IF3, it is of interest to identify those structural elements of the protein which may be responsible for individual functions. Furthermore, it is still an open question whether the many properties of IF3 are the result of a single activity (e.g., ribosome binding) or are individual activities governed by different structural features of the protein. These questions can best be addressed by introducing site-specific alterations in the protein and testing the effects of these changes on IF3 function.

Despite much research on the functional role of IF3 in translation, relatively little is known regarding the structure–function relationships of the protein. Previous chemical modification studies of Gualerzi and co-workers suggested that Y107 and K110 were important for the function of IF3 (Bruhns & Gualerzi, 1980; Ohsawa & Gualerzi, 1981). These experiments involved iodination of the tyrosine residues and modification of the lysine residues by reaction with pyridoxal phosphate. Identification of the specific residue(s) whose modification resulted in loss of function was complicated by the fact that IF3 contains 3 tyrosines and 21 lysines. It was, therefore, of interest to reinvestigate the functional roles of

Y107 and K110 in the initial site-directed mutagenesis studies described here.

Each of the mutant IF3 proteins studied displayed altered 30S binding capacity, with the mutations of K110 yielding a more severe impairment of binding than those at Y107. Removal of the phenol hydroxy group of the tyrosine residue in Y107F resulted in only a 2-fold reduction of binding (Table I). Complete elimination of the aromatic residue by replacement with leucine (Y107L) yielded a further loss in 30S subunit binding capacity (7.8-fold lower relative to wild-type IF3). Mutations of the lysine at position 110 produced more severe reductions in 30S subunit binding—K110R activity being reduced 15-fold. Moreover, its replacement with leucine resulted in an IF3 which lacked any measurable 30S subunit binding capacity.

These results demonstrate that Y107 and K110 are necessary for suitable 30S subunit binding activity, as suggested previously. Bruhns and Gualerzi (1980) interpreted the results of tyrosine iodination experiments as indicating that modification of Y70 had a moderate effect on 30S subunit binding capacity, but the protein was not functional as judged by dissociation of poly(U)–*N*-AcPhe–tRNA–30S subunit ternary complexes (this assay probably tests for the proofreading activity of IF3). Conversely, IF3 iodinated at Y107 was underrepresented in ribosome-bound, modified IF3, which suggested that Y107 modification primarily affected the 30S subunit binding ability of the protein. Reaction of IF3 with pyridoxal phosphate resulted in modification of six lysine residues, and the conclusion was that K110 modification was responsible for the loss of both 30S subunit binding capacity and ternary complex dissociation activity (Ohsawa & Gualerzi, 1981). The present studies shed additional light on the precise structural requirements at these amino acid residues. Thus, maximal binding to 30S subunits requires an aromatic residue at position 107, although relatively strong binding ($K_a$ of $1 \times 10^7$ $M^{-1}$) was observed even in the absence of the ring (Y107L). It would be of interest to determine whether a hydrophobic residue is a requirement at this position, but such mutants have not yet been tested. The nature of the side chain at position 110 appears to be more stringent. Substitution of lysine with arginine resulted in a severe reduction in binding; replacement with leucine (K110L) led to a complete loss of 30S subunit binding ability.

Parallel findings were obtained when the capacity of the Y107 mutants to stimulate 70S ribosome dissociation was analyzed. From these results it is clear that an aromatic group at position 107 is necessary for proper function. The absence of an aromatic side chain in Y107L yielded an IF3 which did not induce measurable dissociation of 70S couples even at 10-fold molar excess of protein. It is of interest to note that *Bacillus stearothermophilus* IF3 has a phenylalanine residue at the position equivalent to Y107 (Kimura et al., 1983). It is generally believed that formation of ribosomal subunits from 70S couples is mediated by IF3 via an antiassociation activity; i.e., spontaneously dissociated 30S subunits have a greatly reduced affinity for 50S subunits by virtue of IF3 binding (Kaempfer, 1972; Gottlieb et al., 1975). There have been reports, however, which indicated that IF3 can play a direct role in 70S ribosome dissociation (Chaires et al., 1982; Goss et al., 1982). It is interesting that there was a correlation between the ability of the Y107 mutants to mediate 70S ribosome dissociation and bind to 30S subunits. Y107F, which displayed only a slight reduction in 30S subunit binding, mediated ribosome dissociation in a manner essentially indistiguishable from wild-type IF3 whereas Y107L, which had a more severe impairment in 30S subunit binding, also had significantly lower dissociation activity.

Recent studies suggest that the critical function of IF3 in initiation is proofreading of the fMet–tRNA–mRNA interaction (Hartz et al., 1989; 1990; Gualerzi & Pon, 1990). The results obtained in the toeprinting assays further support the importance of residues 107 and 110 in IF3 function and extend the findings of Hartz et al. (1989), which indicated that IF3 plays a role in inspecting the incoming tRNA molecules during initiation. It was not clear whether prior IF3–30S subunit complex formation is a prerequiste for proofreading, although it is reasonable to expect that this might be the case. In this regard it is interesting to note that the ability of the mutant IF3s to properly select the $tRNA^{fMet}$ complex over those containing the Phe-tRNA followed very closely their 30S subunit binding capacities (i.e., Y107F > Y107L ≥ K110R > K110L), suggesting that IF3 must be ribosome-bound in order to mediate proofreading.

Recently, Sussman et al. (1990) described the isolation of *E. coli* strains with mutations in *infC*. A number of these mutant alleles appear to alter the IF3 scanning of the initiator tRNA–initiation codon interaction (J. Sussman and R. Simons, personal communication), and the mutation in one of these alleles (*infC*362) has been characterized as a single amino acid change of Y75 to Y75N (D. Liveris, J. Sussman, R. Simons, and I. Schwartz, unpublished results). The ability of plasmid-encoded wild-type Y107 or K110 mutant IF3 to complement the *infC*362 phenotype correlated precisely with 30S subunit affinity. The results indicate that IF3 may play a role in both the selection of initiator tRNA and recognition of the correct tRNA–mRNA initiation complex. Furthermore, the data suggest the existence of at least two structurally distinct domains in IF3—a ribosome binding domain and a proofreading domain which would be involved in initiator tRNA and initiation codon selection. The results presented here indicate that prior binding to ribosomes is a prerequisite for fMet–tRNA selection. However, it is possible that a mutant IF3 with normal ribosome binding properties may be incapable of fMet–tRNA selection. If this were the case, it would establish the existence of separate functional sites for ribosome binding and proofreading. This could be confirmed by demonstration that Y75N IF3 (i.e., IF3 from the *infC*362 allele) is capable of normal ribosome binding activity. Such experiments are currently in progress.

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**Registry No.** Tyr, 60-18-4; Lys, 56-87-1.