The C-terminal Subdomain (IF2 C-2) Contains the Entire fMet-tRNA Binding Site of Initiation Factor IF2*

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Previous protein unfolding studies had suggested that IF2 C, the 24.5-kDa fMet-tRNA binding domain of Bacillus stearothermophilus translation initiation factor IF2, may consist of two subdomains. In the present work, the four Phe residues of IF2 C (positions 531, 599, 657, and 721) were replaced with Trp, yielding four variant proteins having intrinsic fluorescence markers in different positions of the molecule. Comparison of the circular dichroism and Trp fluorescence changes induced by increasing concentrations of guanidine hydrochloride demonstrated that IF2 C indeed consists of two subdomains: the more stable N-terminal (IF2 C-1) subdomain containing Trp-599, and the less stable C-terminal (IF2 C-2) subdomain containing Trp-721. Isolated subdomain IF2 C-2, which consists of just 110 amino acids (from Glu-632 to Ala-741), was found to bind fMet-tRNA with the same specificity and affinity as native IF2 or IF2 C-domain. Trimming IF2 C-2 from both N and C termini demonstrated that the minimal fragment still capable of fMet-binding consists of 90 amino acids. IF2 C-2 was further characterized by circular dichroism; by urea-, guanidine hydrochloride-, and temperature-induced unfolding; and by differential scanning calorimetry. The results indicate that IF2 C-2 is a globular molecule containing predominantly β structures (25% antiparallel and 8% parallel β strands) and turns (19%) whose structural properties are not grossly affected by the presence or absence of the N-terminal subdomain IF2 C-1.

Initiation factor IF2 is the largest (741 amino acids in Bacillus stearothermophilus; Ref. 1) of the three proteins involved in the initiation step of protein synthesis in eubacteria whose main role is that of positioning the initiator fMet-tRNA in the ribosomal P site. To accomplish this function, IF2 interacts with both 30 and 50 S ribosomal subunits and with the initiator tRNA. The latter is one of the most important interactions in the pathway of translation initiation, playing an essential role in the selection of mRNA initiation sites. The specificity of fMet-tRNA recognition by IF2 rests mainly on the presence of the blocked αNH₂ group of methionine, a typically eubacterial feature (2–4). The active site of IF2 involved in the interaction with the 50 S ribosomal subunits is localized, together with the GTP/GDP binding site, in the central domain (G-domain) of the molecule (40 kDa), which is related to the family of GTP/GDP-binding proteins (see Refs. 5–8, and references therein). The 24.5-kDa C-terminal domain (IF2 C) contains the molecular determinants for the recognition and binding of fMet-tRNA (8, 9). This C-domain, obtained as a recombinant protein by genetic manipulation of B. stearothermophilus infB, has been further characterized spectroscopically. Thermal and GuHCl-induced unfolding carried out in this study indicated that IF2 C unfolds in a stepwise process via a stable intermediate, suggesting that this domain may consist of two subdomains (10).

To verify this hypothesis and possibly characterize the subdomains with respect to the primary sequence of IF2 C, in the present study we have introduced intrinsic fluorescence markers at specific sites of the molecule by replacing with Trp residues the four fairly regularly spaced Phe residues of IF2 C; after verifying that the variants were structurally and functionally comparable to the wild type molecule, the fluorescence and CD changes occurring during the unfolding of the four variant proteins were compared. The results demonstrated that IF2 C consists of two subdomains, IF2 C-1 and IF2 C-2, corresponding to roughly the N-terminal and C-terminal halves of the domain molecule, respectively. Isolated IF2 C-2, which contains the last 110 amino acids of IF2, was further characterized structurally and functionally. IF2 C-2 was found to be folded like a stable protein and to contain all the molecular determinants necessary and sufficient for the specific recognition and binding of fMet-tRNA.

**EXPERIMENTAL PROCEDURES**

Buffers—Buffer I consisted of 20 mM Tris-HCl (pH 7.1), 60 mM NH₄Cl, 10 mM MgAc₂, 0.2 mM phenylmethylsulfonyl fluoride. Buffer II consisted of 20 mM Tris-HCl (pH 7.1), 0.1 mM EDTA, 10% glycerol, 5 mM β-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride. Buffer III consisted of 20 mM Tris-HCl (pH 7.1), 0.1 mM EDTA, 5% glycerol, 200 mM NH₄Cl. Buffer IV consisted of 2 mM sodium cacodylate (pH 7.0), 0.5 mM NaCl, 5 mM MgCl₂. Buffer V consisted of 50 mM sodium phosphate buffer (pH 7.2). Buffer VI consisted of 100 mM Tris-HCl (pH 8.0), 160 mM NH₄Cl, 6 mM MgAc₂, 6 mM β-mercaptoethanol.

Chemicals—Ultrapure guanidine hydrochloride (GdnHCl) and urea were from ICN Biomedicals (Cleveland, OH). Sodium cacodylate was from Serva (Heidelberg, Germany). All other chemicals were purchased from E. Merck (Darmstadt, Germany).

Site-directed Mutagenesis—For the Phe → Trp substitutions at designated positions of the IF2 C, the EcoRI-HindIII fragment of infB (1) encoding this domain was excised from pX4041C (9), subcloned in pSELECT, and subjected to site-directed mutagenesis (Altered Sites Mutagenesis System, Promega) using the following mutagenic oligonucleotides: (a) 5'-CGA CGA TTT GTG GGA ACA AAT CAA-3'; (b) 5'-GTC
Amino acids (aa) indicated in italics are derived either from plasmid vectors used for intermediate cloning or from expression vector pEv1 as already described (9). Underlined sequences correspond to restriction sites (EcoRI and HindIII) introduced at the 5’ end of oligonucleotides to facilitate cloning of DNA fragments produced by polymerase chain reaction amplification into expression vector. U and D indicate the position of the oligonucleotide, upstream and downstream of the amplified DNA, respectively.

| Primer sequences | Size and denomination | Amino acid sequence bordering the polypeptide |
|------------------|----------------------|---------------------------------------------|
| U 5'-ggAAAAACGCAGCAGGCGC-3' | (136 aa) | M-N-E-F-E-L-G-T-R-G-S-S-R-V-D-L-515-Q-----------------A-M-551-STOP |
| D 5'-ggCAAgCTTCTACGCGCGCTC-3' | C-1 | M-N-E-F-E-E632-A------------------------R-741-STOP |
| U 5'-ggATTTCAAGGCGCGTGAATA-3' | (114 aa) | M-N-E-F-E-K446-V------------------------R-741-STOP |
| D 5'-ggTTCTGCCTGCTGATGTGTTG-3' | C-2 | M-N-E-F-E-E651-K------------------------R-741-STOP |
| D 5'-ggTCTTTCAAGGTCCGGCAAGAT-3' | 88 aa | M-N-E-F-E-E651-K------------------------R-741-STOP |
| D 5'-ggTCTTCGCTGACTGTTGTTG-3' | C-2 | Δ24N |
| U 5'-ggATTTCAAGGCGCGTGAATA-3' | (108 aa) | M-N-E-F-E-E632-A------------------------V-M-341-STOP |
| D 5'-ggCCCAAGCTTTCTCTACATAGCAGTACGGC-3' | C-2 | Δ6C |
| MURFI linker into Hpal site over Leu74 | (89 aa) | M-N-E-F-E-E632-A------------------------V-T56-STOP |
| U 5'-ggATTTCAAGGCGCGTGAATA-3' | (94 aa) | M-N-E-F-E-K446-V------------------------V-M-341-STOP |
| D 5'-ggCCCAAGCTTTCTCTACATAGCAGTACGGC-3' | C-2 | Δ14N/Δ6C |

ATT GGC TGG AAC GTC GCC CG-3'; (c) 5’-GGC GCA AAC GGA AGT GTC CAA-3'; (d) 5’-ACG ATA AAC TAA GTC GAT GAC AT-3’, for the substitutions at positions 531, 599, 657, and 721, respectively. The mutated EcoRI-HindIII fragments were subsequently recloned in pXP401C, and the corresponding variant proteins were hyperexpressed and purified as described previously for IF2 C (9, 10).

Construction of B. stearothermophilus IF2 C-1 and IF2 C-2 Subdomains—Strains overproducing the two subdomains of B. stearothermophilus IF2 C were prepared by cloning the polymerase chain reaction-amplified targeted DNA region of B. stearothermophilus infB into a plasmid expression vector. Amplification was performed with ULTma tech applying protein samples (14).

The mean residue ellipticities [θ] 218 nm were calculated using the variable κ218 nm assuming absorption coefficients of 0.65 mM⁻¹ cm⁻¹ and 0.69 mM⁻¹ cm⁻¹ for IF2 C mutants and IF2 C-2, respectively, calculated (12) from the amino acid composition, with seven Tyr and one Trp residue in the case of the IF2 C mutants, and with just six Tyr residues in the case of IF2 C-2. Circular Dichroism—CD measurements were performed at 25 °C with a Jasco J-720 spectropolarimeter equipped with a thermostated cell holder and a temperature control system (Neslab) as described (13). The mean residue ellipticities [θ] were calculated using mean residue masses of 110.3 and 113.1 Da for IF2 C Phe → Trp mutants and IF2 C-2, respectively. Far-UV CD spectra were measured in a 0.10-cm or 0.01-cm cuvette at protein concentrations of 0.80 and 0.080 mg/ml, respectively. CD spectra in the near-UV region were recorded in a 1.0-cm cell at protein concentrations of about 2.0 mg/ml. Thermal unfolding of IF2 C-2 in Buffer IV in the presence of various guanidine hydrochloride concentrations was analyzed measuring the ellipticity at 218 nm using a heating rate of 20 °C/h. The secondary structure content was calculated from the far-ultraviolet CD spectra using the variable selection method (program VARSCL1) starting with a set of 33 reference proteins (14).

The latter method sometimes showed the presence of a faster moving band whose N-terminal amino acid sequence indicated that it resulted from the specific cleavage of the acid-sensitive Asp-640→Pro-641 bond, which is significantly more labile in IF2 C-2 compared with IF2 C, indicating an increased accessibility of this bond in the subdomain compared with the whole IF2 C. In agreement with this interpretation, site-directed deletion of Pro-641 resulted in a much more stable protein. Overall, the yield was estimated to be about 4 mg·g⁻¹ of cells.

Sample Preparation—Before spectroscopic and microcalorimetric measurements, the proteins were subjected to gel filtration in Buffer IV on a Superose 12 HR10/30 column (Amersham Pharmacia Biotech). The peak fractions were pooled and exhaustively dialyzed against Buffer IV, Buffer V, or Buffer IV containing GdnHCl at various concentrations. For Raman measurements, the protein solutions were concentrated to about 20 mg/ml using Microcon tubes (Amicon) with 10-kDa cutoff. Protein concentrations were determined from the A278 nm assuming absorption coefficients of 0.65 mM⁻¹ cm⁻¹ and 0.69 mM⁻¹ cm⁻¹ for IF2 C mutants and IF2 C-2, respectively, calculated (12) from the amino acid composition, with seven Tyr and one Trp residue in the case of the IF2 C mutants, and with just six Tyr residues in the case of IF2 C-2.
setup and data analyzed with the software package Spectramax (Jobin-Yvon) supplemented by several in-house programs. Further details have been described elsewhere (15).

**Differential Scanning Calorimetry**—Measurements were performed with a precision scanning calorimeter DASM-1 (Pushchina, Russia) as described earlier (15). A heating rate of 1 K min⁻¹ was kept constant in all experiments. The IF2 C-2 samples were in Buffer IV without and with 0.45, 0.65, and 1.0 M GdnHCl at protein concentrations in the range of 1–1.5 mg ml⁻¹. For data analysis and deconvolution, the Origin for DSC software package (MicroCal, Northampton, MA) was used, providing half transition temperature Tm and enthalpy changes ΔHm. Heat capacity change ΔCp for DSC software package (MicroCal, Northampton, MA) was used, providing free energy changes ΔGm between native and unfolded state (N→U), and free energy change ΔGf at 25 °C were calculated according to standard equations (Equations 1–3).

\[ \Delta C_p = \frac{d(\Delta H)/dT}{T} \]

\[ \Delta S_m = \frac{\Delta H_m}{T_m} \]

\[ \Delta G_m(T) = \Delta G_m(1 - T/T_m) + \Delta C_p(T - T_m) + T \ln (T/T_m) \]

**Equilibrium Unfolding and Refolding Studies**—Denaturation-dependent conformational transitions were monitored measuring changes of the fluorescence spectra and of the circular dichroism at 220 or 218 nm. Separate samples for each point were made with a protein concentration of 0.080 mg ml⁻¹. For refolding experiments, IF2 C mutants and IF2 C-2 were first unfolded by incubation in the presence of 7 M GdnHCl for at least 6 h at room temperature and then refolded by addition of renaturation buffer. All solutions were incubated overnight at room temperature and were measured at 25 °C. Concentration of GdnHCl stock solution in Buffer IV was determined from refractive index measurements, and the fraction of unfolded protein was calculated as described by Pace and Schultz (16). Assuming “two-state” transitions of unfolding the free energy changes ΔGm between native and intermediate state (N→I) and ΔGf between intermediate and unfolded state (I→U) of IF2 C mutants, and the free energy change ΔGf at 25 °C were calculated as described (10).

**Activity Tests**—The activity of intact IF2, of IF2 C (wild type and mutants), and of IF2 C-2 in binding fMet-tRNA was measured quantifying the protection conferred by these proteins on the initiator tRNA. Reaction mixtures (50 μl) in Buffer VI contained 22 μM f[35S]Met-tRNA and the indicated concentrations of the proteins to be tested. Samples (20 μl), withdrawn after 0 and 60 min of incubation at 37 °C, were spotted on Whatman 3MM paper discs for determination of the cold trichloroacetic acid-insoluble radioactivity.

**RESULTS**

As mentioned in the Introduction, unfolding studies carried out on B. stearo thermophilus IF2 C suggested that this domain may consist of two subdomains with different stability. To confirm this premise and possibly identify the regions of IF2 C corresponding to the two subdomains, we decided to compare the global CD changes occurring during IF2 C unfolding with the fluorescence changes induced by the same process originating from intrinsic fluorescence markers introduced at specific sites of the molecule. Considering that IF2 C contains no Trp residues and only four Phe residues regularly spaced (positions 531, 599, 657, and 721), we replaced these four Phe residues with Trp, thus producing four variant proteins.

**Preparation, Purity, and General Properties of the IF2 C Mutant Proteins**—The IF2 C F531W, IF2 C F599W, IF2 C F657W, and IF2 C F721W mutants, constructed, expressed in E. coli, and purified to at least 95% homogeneity (as described under “Experimental Procedures”), contain 222 amino acids corresponding to a calculated molecular mass of 24491 g mol⁻¹. Heat capacity change ΔCp corresponding to Ser-520 of native IF2 C at their N terminus and, like wt IF2 C (10), behave as monomers on a Superose 12 column in Buffer IV. Aside from some minor differences detected in the spectra of IF2 C F599W and IF2 C F721W, their far-UV and near-UV CD spectra (data not shown) are almost superimposable on those of wt IF2 C (10), indicating that they all have the same type of folding and secondary structure. Our results suggested, however, that the Trp residue in position 721 has a somewhat lower mobility and higher asymmetry compared with the Trp residues introduced in the other three positions. Similar indications are also given by Raman spectroscopy of the mutant proteins.

**Characterization of the IF2 C Mutants by Raman Spectroscopy**—Aside from the expected presence of the additional bands due to the Trp residues, the Raman spectra of the four mutant proteins (data not shown) are similar to that of wt IF2 C (10). The lack of significant spectral differences involving the amide I bands near 1667 cm⁻¹ indicates conservation of the general protein fold in all IF2 C mutants. Furthermore, the identical intensity ratio of the Tyr doublet at 828 and 854 cm⁻¹ (I828/I854 = 1.83 ± 0.02) found for the mutants F531W, F599W, and F657W and for wt IF2 C indicates that in these proteins the Tyr residues have similar properties and are found in similar environments; the slightly higher ratio (1.92) found in the F721W mutant, on the other hand, indicates that the Tyr residues of this protein are in a slightly more hydrophilic environment and/or that one of them is involved in the formation of a strong H bond.

**Biological Activity of IF2 C Mutants**—The only biological activity characteristic of wt IF2 C is that of recognizing and binding fMet-tRNA with the same specificity and affinity as the complete IF2 molecule (9). Functional tests carried out with the four mutants demonstrated that, compared with wt IF2 C, IF2 C F531W and IF2 C F657W are fully active, IF2 C F721W slightly less active, and the F599W mutant is 3-fold less active in initiator tRNA binding (data not shown). Taken together, the preliminary characterization of the four IF2 C mutants indicates that none of the four Phe residues of IF2 C plays an essential role in the recognition and binding of initiator tRNA and that, as expected from the conservative nature of the amino acid replacements, none of the individual Phe→Trp substitutions grossly alter the structure or function of IF2 C.

Thus, we concluded that all these variants, including the F721W mutant, which exhibits some minor structural and functional anomalies, can be used as models to monitor the unfolding behavior of IF2 C.

**Fluorescence Spectra and GdnHCl-induced Fluorescence Changes**—Since fluorescence changes accompanying the GdnHCl-induced unfolding of a protein depend on the individual properties of the tryptophan fluorophor(s), which in turn depend on the hydrophobicity of their environment, the tryptophan residues at positions 599 and 721 seemed to be the most suitable fluorescence markers to follow the process of protein unfolding. In fact, the emission maxima of both Trp-599 and Trp-721 (upon Trp excitation at 295 nm) were found to be blue-shifted (at 332 nm) compared with those of Trp-531 and Trp-657 (both at 348 nm) and KI quenching, quantified by Stern-Volmer plots (data not shown), was found to be in the order F657W > F531W > F599W > F721W, indicating that Trp-599 and Trp-721 are located in a more hydrophobic and less accessible environment than Trp-531 and Trp-657. As predicted, the emission maxima of the buried tryptophan residues (Trp-599 and Trp-721) undergo a red-shift (from 332 nm to about 350 nm) during GdnHCl-induced protein unfolding (Fig. 1A), while, under the same conditions, Trp-531 and Trp-657 undergo a large increase in fluorescence intensity (≈100%), which is accompanied by a negligible (≤2 nm) red-shift (Fig. 1A).

**GdnHCl-induced Unfolding of the IF2 C Phe→Trp Mutants**—As seen in Fig. 1A, when GdnHCl-induced unfolding was analyzed measuring changes of the fluorescence emission maxima, which monitor the environment of each tryptophan residue, a single transition was observed for both F599W and F721W IF2 C mutants. This is in contrast to the two-step
fMet-tRNA-IF2 Interaction

TABLE II

| Protein     | Monitored signal | $\Delta G_{\text{H}2\text{O}}^{\circ}$ | $m_{\text{NI}}$ | $c_{\text{m,NI}}$ | $\Delta G_{\text{H}2\text{O}}^{\circ}$ | $m_{\text{IU}}$ | $c_{\text{m,UI}}$ |
|-------------|------------------|-------------------------------|----------------|-----------------|-------------------------------|----------------|-----------------|
| WT$^a$      | $\Theta_{220\text{nm}}$ | 6.2                          | 3.0            | 2.05            | 12.9                         | 3.1            | 4.14            |
| F721W      | $\Theta_{220\text{nm}}$ | 7.6                          | 2.9            | 2.67            | 12.8                         | 3.1            | 4.15            |
| F599W      | $\lambda_{\text{max}}$ | 6.9                          | 2.6            | 2.96            | 10.0                         | 2.8            | 3.55            |
| F657W      | $\lambda_{\text{max}}$ | 6.6                          | 2.8            | 2.36            | 10.3                         | 3.0            | 3.39            |
| F599W      | $I_{360\text{nm}}$  | 9.2                          | 2.7            | 2.42            | 12.8                         | 3.1            | 4.15            |
| F531W      | $\Theta_{220\text{nm}}$ | 5.4                          | 2.5            | 2.20            | 11.5                         | 2.8            | 4.14            |
| F657W      | $\Theta_{220\text{nm}}$ | ND                           | ND             | ND              | 10.2                         | 2.5            | 4.06            |
| F599W      | $\Theta_{220\text{nm}}$ | 3.0                          | 1.7            | 1.73            | 11.5                         | 2.8            | 4.14            |

$^a$ Monitored signals: $\Theta_{220\text{nm}}$, ellipticity at 220 nm; $\lambda_{\text{max}}$, fluorescence maximum; $I_{360\text{nm}}$, fluorescence intensity at 360 nm. Units: $\Delta G_{\text{H}2\text{O}}^{\circ}$, kcal mol$^{-1}$; $m$, kcal mol$^{-1}$ cm$^{-1}$; $c_{\text{m}}$ and $m_{\text{m}}$, cooperativity parameter of the unfolding transition; $c_{\text{m}}$, GdnHCl transition midpoint concentration. Two-state transitions were assumed from the native to an intermediate state and from the intermediate to the unfolded state. $\Delta G_{\text{H}2\text{O}}^{\circ}$ and $\Delta G_{\text{H}2\text{O}}^{\circ}$ are the free energy differences (at 25 °C) between native and intermediate (one folded and one unfolded domain), and between intermediate and unfolded states, respectively.

Cloning, Hyperproduction, and Purification of IF2 C-2—The above data demonstrate that, as previously suggested by Miseiwitz et al. (10), IF2 C indeed consists of a more stable N-terminal subdomain (IF2 C-1) containing Trp-599 and of a more labile C-terminal subdomain (IF2 C-2) containing Trp-721. Since the hydropathic properties of IF2 C indicate the presence of a marked hydrophobic (the N-terminal half) and of a hydrophilic C-terminal half, we next sought to establish whether subdomains IF2 C-1 and IF2 C-2 coincide with these two regions of different hydropathicity, whether the two subdomains can exist as isolated molecules, and whether either one may retain the site responsible for fMet-tRNA binding.

To this end, following the genetic manipulations described under “Experimental Procedures,” we cloned the relevant portions of the infB gene to construct two vectors each expressing a different protein fragment: the N-terminal half and the C-terminal half of the C-domain corresponding to the putative IF2 C-1 and IF C-2 subdomains (see scheme in Fig. 2). Although both IF2 C-1 and IF2 C-2 fragments were readily produced, the results initially obtained with IF2 C-2 prompted us to devote our efforts exclusively toward the functional and structural characterization of this fragment.

General Properties and fMet-tRNA Binding Capacity of IF2 C-2—Subdomain IF2 C-2, purified according to the protocol described under “Experimental Procedures,” is a protein containing a total of 114 residues, of which the N-terminal 4 (M-N-E-F) and the C-terminal 110 residues (M-L-N-L-A-A-L-A-A-A-L-N-L-A-A-L-A) derive from the cloning procedure (Table I and Fig. 2).

The capacity of IF2 C-2 to interact with fMet-tRNA was measured as the ability of the protein to protect the acyl bond of the initiator tRNA from hydrolysis catalyzed by Tris ions at high pH. Comparison of IF2 C-2 with native IF2 demonstrates that these proteins have the same affinity ($\Delta G_{\text{H}2\text{O}}^{\circ} = 9.0$ kcal mol$^{-1}$) and the same specificity for the initiator tRNA. In fact, increasing concentrations of intact IF2 and of IF2 C-2 protect the same amounts of fMet-tRNA, giving rise to almost identical titration curves (Fig. 3A), while dilution with increasing amounts of

FIG. 1. Denaturant-induced unfolding of IF2 C Phe → Trp variants. A, GdnHCl-induced unfolding of the four IF2 C Phe → Trp variants monitored through changes of the Trp emission maxima following excitation at 295 nm: IF2 C F531W (△), IF2 C F657W (○), IF2 C F599W (●), and IF2 C F721W (□). B and C, normalized GdnHCl-induced unfolding transition curves of IF2 C F721W (B) and IF2 C F599W (C) mutants showing the fraction of unfolded protein (ordinate) monitored by changes of the ellipticity at 220 nm (filled symbols) and the shift of the emission maximum (open symbols) as a function of the denaturant concentrations indicated in the abscissa. All measurements were performed at 25 °C in Buffer IV containing the indicated concentrations of GdnHCl at a protein concentration of 0.080 mg ml$^{-1}$.

transition observed with wt IF2 C (10) and with both F721W (Fig. 1B) and F599W (Fig. 1C) IF2 C variants when the same process was monitored by the ellipticity changes (at 220 nm), which stem from general modifications of the secondary structure. Comparison of the normalized CD (filled symbols) and fluorescence (open symbols) unfolding transition curves of IF2 C F721W (Fig. 1B) and of IF2 C F599W (Fig. 1C) clearly indicates that the transitions monitored by Trp-721 and Trp-599 correspond to the first and to the second transition mon-
buffer of the complexes formed by these proteins with fMet-tRNA causes a similar decrease in the level of protected fMet-tRNA (Fig. 3B). Furthermore, variations in Mg\textsuperscript{2+} (Fig. 3C) and NH\textsubscript{4}\textsuperscript{+} (Fig. 3D) concentrations influenced in similar ways the fMet-tRNA protection by intact IF2 and by IF2 C-2. Finally, intact IF2, the whole IF2 C domain and subdomain IF2 C-2 protect equally well formylated Met-tRNA\textsuperscript{fMet} (Fig. 3E) and NacPhe-tRNA (Fig. 3F), while displaying no detectable affinity for either non-formylated Met-tRNA\textsuperscript{nMet} (Fig. 3E) or non-acetylated Phe-tRNA (data not shown). Taken together, these data allowed us to conclude that IF2 C-2 contains the entire active site of IF2 responsible for the recognition and binding of initiator tRNA and that the activity of this site is unaffected by the presence or absence of the remaining portions of the molecule.

**Structural Characterization of IF2 C-2**—In light of the above premises, we pursued a structural characterization of IF2 C-2 and compared its properties with those of the whole IF2 C domain. The CD spectra in the peptide region (182–260 nm) and the content of secondary structures thereby calculated (Table III) indicate that, compared with IF2 C-1 and IF2 C-2, which have been hyperproduced. The dotted lines extending from each thick bar representing a domain or subdomain represent amino acids derived from the genetic manipulations. The numbers in parentheses refer to the size (in kDa) of each protein fragment. The N- and C-terminal amino acids of each fragment are also indicated.

![Diagram illustrating the domain structure of B. stearothermophilus IF2, the subdomain structure of its C-domain (IF2 C), and the region of the molecule corresponding to subdomains IF2 C-1 and IF2 C-2, which have been hyperproduced.](image)

**Fig. 2. Domain structure of IF2 C.** Diagram illustrating the domain structure of B. stearothermophilus IF2, the subdomain structure of its C-domain (IF2 C), and the region of the molecule corresponding to subdomains IF2 C-1 and IF2 C-2, which have been hyperproduced. The dotted lines extending from each thick bar representing a domain or subdomain represent amino acids derived from the genetic manipulation procedures. The numbers in parentheses refer to the size (in kDa) of each protein fragment. The N- and C-terminal amino acids of each fragment are also indicated.

**Fig. 3. Comparison of the aminoacyl-tRNA binding activity of native IF2 (○), domain IF2 C (•), and subdomain IF2 C-2 (●).** fMet-tRNA protection as a function of: A, increasing concentrations of IF2 and IF2 C-2; B, increasing dilutions of the binary complexes containing fMet-tRNA and either IF2 or IF2 C-2; C, increasing concentrations of Mg\textsuperscript{2+}; and D, NH\textsubscript{4}\textsuperscript{+} in the presence of IF2 or IF2 C-2. E, specificity of the interaction of IF2, IF2 C, and IF2 C-2 with formylated (continuous line) versus non-formylated (dotted line) Met-tRNA\textsuperscript{fMet}; F, comparison of the NacPhe-tRNA protection by IF2, IF2 C, and IF2 C-2. When appropriate (B–D), the fMet-tRNA remaining intact at the end of the incubation in the absence of protein is indicated □. Further details are given under “Experimental Procedures.”

**Temperature-induced Unfolding and Differential Scanning Calorimetry of IF2 C-2**—Changes of the ellipticity at 218 nm were monitored to measure the thermal unfolding of IF2 C-2. In Buffer IV containing 0.4, 0.62, 0.88, and 1.1 M GdnHCl, half-transition temperatures of 78.2, 74.0, 69.0, and 62.5 °C, respectively, were obtained (data not shown). The T\textsubscript{m} values decrease linearly with increasing GdnHCl concentrations, and
extrapolation to 0 M GdnHCl gives a 
T_m 
of 88 °C.

Difference heat capacity curves are shown in Fig. 5
A.

T_m 
values of 88.1, 81.8, 78.8, and 74.1 °C were obtained in Buffer
IV without GdnHCl and with 0.45, 0.65, and 1.0 M GdnHCl,
respectively (Fig. 5
B). The enthalpy differences 
\( \Delta H_m \) depend linearly on the GdnHCl concentration (Fig. 5
C). Quotients \( k \) between calorimetric (\( \Delta H_{cal} \)) and van’t Hoff (\( \Delta H^{TH} \)) enthalpy

TABLE III
Secondary structure content of IF2 C-2 (in %)
The content of secondary structure was calculated with the program
VARSLC1 (14).

|                | Helix | \( \beta \)-Sheet | Turn | Remainder
|----------------|-------|------------------|------|---------|
| IF2 C wt\(^a\) | 25 (± 1) | 23 (± 2) | 8 (± 0) | 16 (± 1) | 29 (± 1) |
| IF2 C-2\(^b\) | 11 (± 1) | 25 (± 1) | 8 (± 1) | 19 (± 1) | 37 (± 1) |

\(^a\) Remainder is the difference between the sum of helix, \( \beta \)-sheet and turn, and 100% secondary structure.

\(^b\) Values were taken from Ref. 10.

\(^c\) Buffer V.

A

B

C

Fig. 4. CD spectra and denaturant-induced unfolding of IF2 C-2 and IF2 C. A, far-UV CD spectra of IF2 C-2 (1 and 2) and IF2 C (3) recorded at 25 °C at 0.8 mg ml\(^{-1}\) protein concentrations in Buffer IV (2) and in Buffer V (1 and 3). Further details are given under “Experimental Procedures.” B, normalized transition curves of GdnHCl-induced unfolding (\( \triangle \)) and refolding (\( \bigtriangleup \)) of IF2 C-2 were obtained monitoring changes of the ellipticity at 218 nm. GdnHCl-induced unfolding was also measured monitoring the fluorescence intensity at 302 nm (\( \ast \)). As a reference is shown the GdnHCl-induced unfolding curve of IF2 C (\( \circ \)) measured by CD at 220 nm (10). The measurements were performed at 25 °C in Buffer IV at a protein concentration of 0.080 mg ml\(^{-1}\).

FIG. 5. Thermal-induced unfolding of IF2 C-2 in the presence of GdnHCl. A, experimental difference heat capacity curves of IF2 C-2 at 0, 0.45, 0.65, and 1.0 M GdnHCl in Buffer IV. B and C, plots of half-transition temperature (\( T_m \)) (B) and enthalpy change \( \Delta H_m \) versus GdnHCl (C).

TABLE IV
Thermodynamic parameters of IF2 C-2

|            | \( T_m \) | \( \Delta H_m \) | \( \Delta C_p \) | \( \Delta S_m \) | \( \Delta G_{25} \) | \( \Delta G_{25}^a \) |
|------------|---------|----------------|----------------|--------------|----------------|----------------|
| °C         | kcal/mol\(^{-1}\) | kcal/mol\(^{-1}\) K\(^{-1}\) | cal/mol\(^{-1}\) K\(^{-1}\) | kcal/mol\(^{-1}\) | kcal/mol\(^{-1}\) |
| 88.0       | 92.6    | 1.56           | 256            | 7.1          | 5.6            |

\(^a\) Average of the spectroscopic determinations (unfolding at 25 °C with GdnHCl and urea, CD, and fluorescence monitoring).

differences are close to 1 (between 0.86 and 0.94) and provide evidence for the two-state mechanism of the unfolding of IF2 C-2. With the equations given under “Experimental Procedures,” \( \Delta C_p \), \( \Delta S_m \), and \( \Delta G_{25} \) were calculated (Table IV). The data indicate a rather high thermostability and a moderate free energy of unfolding \( \Delta G_{25} \) for IF2 C-2. The difference of 1.5 kcal mol\(^{-1}\) between the calorimetrically and spectroscopically determined value of \( \Delta G \) is probably caused by methodological problems in calculations, since both methods used include rather far extrapolation procedures.

\( f \)Met-tRNA-IF2 Interaction

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The Minimal IF2 Fragment with fMet-tRNA Binding Capacity—Having established that IF2 C-2 is a stable, structured subdomain, which contains the entire fMet-tRNA binding site of IF2, we carried out experiments aimed at defining the size and nature of the shortest polypeptide fragment still capable of fMet-tRNA recognition and binding. For this purpose the DNA segment encoding IF2 C-2 was subjected to “trimming” from either promoter distal or promoter proximal end as well as from both ends, taking care to maintain the correct reading frame (for further details, see “Experimental Procedures” and Table 1). These manipulations resulted in the production of IF2 C-2 subdomains that were somewhat shorter at their N terminus and/or C terminus according to the scheme presented in Fig. 6A. Each protein fragment was overexpressed, purified, and tested for its fMet-tRNA binding capacity. As seen in Fig. 6B, shortening by 14 amino acids at the N terminus and/or 6 at the C terminus yielded proteins with the same activity as IF2 C-2. Since additional trimming of a total of 25 amino acids at either N or C terminus yielded protein fragments completely inactive (the Δ25N) or completely insoluble (the Δ25C), we can conclude that the entire fMet-tRNA binding domain of B. stearothermophilus translation initiation factor IF2 is contained within a 90-amino acid fragment spanning from Lys-646 through Met-735.

DISCUSSION

Previous studies had shown that IF2 C, the 24.5-kDa C-terminal domain of IF2, is a compact structural-functional unit that contains the fMet-tRNA binding site of the molecule and is remarkably resistant to the action of several proteolytic enzymes, at least under normal conditions of pH, temperature, and ionic strength (8, 9). A subsequent study revealed, however, that IF2 C unfolds in two discrete steps upon exposure to denaturing agents and can be cleaved in two fragments by proteolytic digestion at acidic pH; these results and a hydrophathy profile revealing the presence of a fairly hydrophobic N-terminal half and of a hydrophilic C-terminal portion led us to suggest the possibility that IF2 C may be constituted by two subdomains (10).

With the ultimate goal of characterizing better the mechanism by which fMet-tRNA is recognized and bound by IF2, in the present study we sought to determine whether IF2 C indeed consists of two subdomains and, if so, to identify their nature. The experimental approach undertaken was to replace by site-directed mutagenesis the four Phe residues of the molecule with Trp residues, to verify that these conservative amino acid replacements had not grossly altered either structure or function of the resulting variants, and finally to compare the changes in the CD signals and in the Trp fluorescence emission caused by GdnHCl-induced unfolding.

Correlation of Trp-657 and Trp-531 fluorescence changes with the unfolding process was not possible, since these residues were found to be localized on the surface and changes of their fluorescence intensity did not provide information about unfolding of the corresponding regions of the molecule. On the contrary, Trp-721 and Trp-599 were found to be located more internally and monitoring their fluorescence provided clear evidence for the localization of these residues in two different folding units of the molecule, Trp-721 in the more labile one, which therefore corresponds to the C-terminal region of the protein (IF2 C-2); and Trp-599 in the more stable subdomain, corresponding to the N-terminal region (IF2 C-1). After establishing this fact, we manipulated the infB gene to obtain the inducible hyperexpression of the two subdomains of IF2 C and characterized their properties. This work demonstrated that purified IF2 C-2 binds initiator tRNA in the same way and with the same specificity as the whole IF2 molecule, allowing for the conclusion that the last 110 C-terminal amino acids of IF2 contain all structural elements necessary and sufficient for the recognition and binding of fMet-tRNA. Further experiments aimed at defining the minimal protein size still capable of fMet-tRNA binding demonstrated that the active site of IF2 is fully contained in an even smaller fragment consisting of only 90 amino acid residues. The structural characterization of IF2 C-2 that ensued from these findings demonstrated that this subdomain is essentially a β-protein endowed with a compact and folded structure, which readily renatures upon GdnHCl-induced unfolding and which is not grossly affected by the presence of the remaining portions of the IF2 molecule. The latter finding offers a structural explanation for the fact that binding of GTP/GDP to IF2 (which occurs in the central G-domain of the molecule) does not influence the interaction of this factor with initiator tRNA (17, 18). This situation and the fact that IF2 requires only a small portion of its C-terminal domain, which constitutes a structurally and functionally independent subdomain to bind the initiator fMet-tRNA, clearly distinguish IF2 from EF-Tu. In fact, the interaction of the elongation factor with aminoacyl-tRNAs is strongly dependent on GTP and requires the participation of all three structural domains (I, II, and III) of this protein (19, 20). Throughout the years, the general tendency has been that of considering IF2 a sort of specialized brand of EF-Tu, and many have tried to pinpoint real or sometimes imaginary structural and functional homologies between these proteins (for a review, see Ref. 4). Our present data indicate that, beyond some undeniable structural resemblance, IF2 and EF-Tu have rather different structural and functional organizations.
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