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Preliminary characterization by X-ray diffraction and Raman spectroscopy of a crystalline complex of *Bacillus stearothermophilus* initiation factor 2 C-domain and fMet-tRNA^{fMet}

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Bacillus stearothermophilus translation initiation factor 2 (IF2) specifically binds initiator fMet-tRNA^{fMet} and positions it into the ribosomal peptidyl site in the course of the initiation of protein biosynthesis. The isolated C-terminal domain of IF2 is capable of binding fMet-tRNA^{fMet}, as shown by RNase A and hydrolysis protection experiments. In the presence of fMet-tRNA^{fMet}, the IF2 C-domain yielded orthorhombic crystals of space group $I222$ ($I2_12_12_1$) diffracting to 3.4 Å resolution. The existence of equimolar amounts of tRNA and protein in the crystals was proven by Raman spectroscopy. The observed unit cell suggests the presence of two IF2 C-domain–fMet-tRNA^{fMet} complexes per asymmetric unit of the crystal.

1. Introduction

Ribosomal protein biosynthesis can be divided in three main steps: initiation, elongation and termination of translation. In prokaryotes, mRNA-binding initiation factor 2 (IF2) and initiator fMet-tRNA^{fMet} association occurs at the 30S ribosomal subunit. First, the Shine–Dalgarno interaction between nucleotides at the 5′-end of the mRNA and the 3′-end of 16S rRNA takes place and the start codon of the mRNA is then positioned into the ribosomal P-site, while IF2 in complex with GTP, which is bound to the 30S ribosomal subunit, binds fMet-tRNA^{fMet}. After ribosome-induced activation of the intrinsic GTPase activity of IF2, hydrolysis of the IF2-bound GTP to GDP and phosphate, and association of the 30S and 50S ribosomal subunits, the fMet-tRNA^{fMet} is correctly positioned into the P-site of the programmed 70S ribosome by IF2 (Gualerzi & Pon, 1990). The precise mechanism of initiation, the role of GTP hydrolysis, the specificity of the IF2–fMet-tRNA^{fMet} interaction and its exact function at the ribosomal P-site, however, are not completely understood.

The C-terminal domain of *Bacillus stearothermophilus* IF2 (IF2-C) specifically interacts with fMet-tRNA^{fMet} and is responsible for complex formation with this molecule (Gualerzi & Pon, 1990; Gualerzi *et al.*, 1991). It is relatively stable to protease digestion. Sequence comparison of the C-terminal parts of different prokaryotic IF2 proteins reveals high similarity (Spurio *et al.*, 1993). IF2-C alone (24 kDa) can complex initiator fMet-tRNA^{fMet}. Until now, neither the three-dimensional structure of IF2 nor of its complex with fMet-tRNA^{fMet} were available. Three-dimensional structures are known for the two other prokaryotic translation initiation factors, IF1 (Sette *et al.*, 1997) and IF3 (Biou *et al.*, 1995).

2. Materials and methods

IF2 and IF2-C from *B. stearothermophilus* were purified according to Gualerzi *et al.* (1991). tRNA^{fMet} was isolated chromatographically from *Escherichia coli* tRNA^{bulk} (Biogenes, Berlin). 17000 A_{260} units tRNA^{bulk} were applied to a Sepharose 6B column (Pharmacia) in 20 m*M* NaOAc pH 5.0, 10 m*M* $MgSO_4$ and 2 *M* $(NH_4)_2SO_4$. tRNAs were eluted in the same buffer with a linear reverse salt gradient from 2 to 0 *M* $(NH_4)_2SO_4$ at room temperature. tRNA-containing fractions were assayed for tRNA^{fMet} activity as described below. After desalting by dialysis and following Biogel P6 (BioRad) chromatography, tRNA^{fMet} was further purified by anion-exchange column chromatography. A Sephadex A 50 column (Pharmacia) was equilibrated in 20 m*M* Tris–HCl pH 7.5, 10 m*M* $MgCl_2$ and 375 m*M* NaCl at room temperature. 2200 A_{260} units of the tRNA^{fMet}-enriched Sepharose 6B pool were applied to the Sephadex A 50 column, eluted isocratically in the buffer described above and assayed for tRNA^{fMet} activity. We obtained 600 A_{260} units of purified tRNA^{fMet} with a specific activity of 1550 pmol methionine per A_{260} unit of tRNA^{fMet}. Met-tRNA^{fMet} could be formylated completely as described below.

Aminoacylation and formylation of tRNA^{fMet} occurred in 50 m*M* HEPES/KOH pH 7.6, 150 m*M* KCl, 12.5 m*M* $MgCl_2$, 2.5 m*M* β-mercaptoethanol, 10 m*M* ATP, 1–50 µ*M*

Table 1
Crystal parameters for IF2-C–fMet-tRNA^{fMet} (mercury derivative).

Crystal size (mm)	0.6 × 0.3 × 0.3
Space group	$I222/I2_12_12_1$
Unit-cell parameters (Å)	
a	74.7
b	116.2
c	227.9
V (Å^3)	1.98 × 10^6
Molecular weight of complex (kDa)	50
V_m (Å^3 Da^{-1})	2.47
Complexes per asymmetric unit	2

tRNA^{fMet}, 20 μ*M* (^{14}C)-methionine (specific radioactivity 50 Ci mol^{-1}; Amersham Buchler, Karlsruhe or New England Nuclear, Boston) for analytical tests or with 1 m*M* methionine for preparative aminoacylation. 1 m*M* N^{10}-formyl tetrahydrofolate was added as a formyl donor. An *E. coli* enzyme preparation which included methionyl-tRNA synthetase and N^{10}-formyl tetrahydrofolate formyl transferase was either purchased from Biogenes (Berlin) or prepared from a nucleic acid-free and ribonuclease-free *E. coli* S100 fraction. The reaction mixture was incubated at 310 K. For analytical tests, incubation took up to 10 min and the aminoacylation was determined by precipitation of the radioactively labelled (^{14}C)-fMet-tRNA^{fMet} with 5% aqueous trichloroacetic acid and subsequent scintillation counting. The extent of formylation was examined according to Schofield & Zamecnik (1968) and Schofield (1970). For preparative aminoacylation, the reaction was incubated for 60 min at 310 K, the fMet-tRNA^{fMet} was purified from the reaction mixture by phenol and ether extraction and ethanol precipitation. fMet-tRNA^{fMet} was redissolved in 10 m*M* HEPES/KOH pH 6.6, 1 m*M* $MgCl_2$.

IF2-GTP or IF2-C were complexed with fMet-tRNA^{fMet} in 20 m*M* HEPES/KOH pH 7.5, 10 m*M* $MgCl_2$, 200–500 m*M* KCl, 1 m*M* GTP or GppNp for IF2 or in 10 m*M* HEPES/KOH pH 6.6, 1 m*M* $MgCl_2$ for IF2-C. The molar ratio of protein to tRNA was 1:1 for crystallization experiments or with a variable excess of protein for biochemical experiments. The complex was concentrated by filtration over a membrane with an exclusion limit of 10 kDa (Amicon).

Hydrolysis protection of the aminoacyl ester linkage (Pingoud *et al.*, 1977) of fMet-tRNA^{fMet} by IF2-GTP or IF2-C was examined in 100–200 μl 150 m*M* Tris–HCl pH 7.5, 75 m*M* NH_4Cl, 10 m*M* $MgCl_2$ and 5 m*M* β-mercaptoethanol, with 2.5 μ*M* (^{14}C)-fMet-tRNA^{fMet} and a protein excess of 1- to 30-fold. For IF2, 200 m*M* KCl and 1 m*M* GTP or GppNHp were added. Incubation took place at 310 K. The rate of deacylation was examined by trichloroacetic acid precipitation and subsequent scintillation counting.

Ribonuclease protection by IF2 proteins was assayed as described by Tanada *et al.* (1981) and modified by Louie & Jurnak (1985), using the same buffers and concentrations as described for the hydrolysis-protection experiments, with the exception that incubation took place on ice. The ribonuclease cleavage reaction was initiated by the addition of 10 μg ribonuclease A per reaction mixture. The reaction was stopped by the addition of 4.5 nmol of tRNA^{bulk} and the extent of (^{14}C)-fMet-tRNA^{fMet} digestion was determined by measuring the trichloroacetic acid insoluble radioactivity.

For gel-retardation experiments, tRNA^{fMet} was aminoacylated with a mixture of (^{35}S)-methionine at a specific radioactivity of 2 Ci mmol^{-1}. Complexes with IF2-C were formed in 50 m*M* HEPES/KOH pH 7.5, 10 m*M* $MgCl_2$, 50 m*M* NH_4Cl at a (^{35}S)-fMet-tRNA^{fMet} concentration of 1 μ*M* and different molar excesses of IF2-C. The gel system consisted of 10%(*w*/*w*) acrylamide, 0.275%(*w*/*w*) bisacrylamide, 20 m*M* MOPS/NaOH pH 7.5. Electrophoresis gels were run for 3 h at 100 V and room temperature and the buffer contained 20 m*M* MOPS/NaOH pH 7.5. The gel was dried for 2 h at 353 K and autoradiography took 12–24 h. Assuming 1:1 stoichiometry, the apparent dissociation constant of the IF2-C–(^{35}S)-Met-tRNA^{fMet} complex was estimated by calculating the concentration of free and complexed (^{35}S)-fMet-tRNA^{fMet} at a 50% distribution of the band corresponding to the free aminoacylated tRNA and the shifted band corresponding to the (^{35}S)fMet-tRNA^{fMet} in complex.

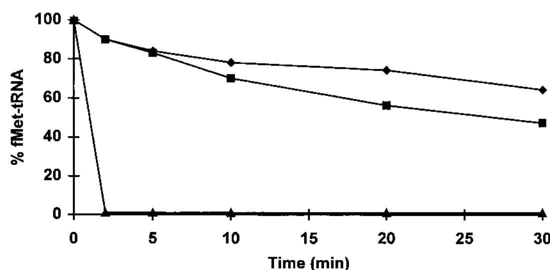

Figure 1
Ribonuclease protection of fMet-tRNA^{fMet} complexed to *B. stearothermophilus* IF2–GTP and IF2-C. Complex formation with 2.5 × 10^{-6} *M* (^{14}C)-fMet-tRNA^{fMet} and a 20-fold molar excess of either IF2–GTP (◆) or IF2-C (■). (^{14}C)-fMet-tRNA^{fMet} without the addition of IF2 was taken as a control (▲). Ribonuclease A was added at *t* = 0 and the cleavage of (^{14}C)-fMet-tRNA^{fMet} was monitored for 30 min. The initial concentration of (^{14}C)-fMet-tRNA^{fMet} was set to 100%.

For crystallization, a complex of IF2-C and fMet-tRNA^{fMet} was prepared as described above with a 1:1 molar ratio of protein and tRNA in 10 m*M* HEPES/KOH pH 6.6 and 1 m*M* $MgCl_2$ at a concentration of 2.25 × 10^{-4} *M*. Initial crystallization screening was carried out by hanging-drop vapour-diffusion methods according to Jancarik & Kim (1991) and Cudney *et al.* (1994) at 277, 291 and 303 K. The drops were prepared by mixing 1 μl of IF2-C–fMet-tRNA^{fMet} with an equal volume of precipitant solution. 1%(*v*/*v*) polyethylene glycol 400 as an additive prevented attachment of crystals to surfaces. Sitting-drop vapour-diffusion methods and macro-seeding procedures were applied to maximize crystal size. Mercury was introduced into the crystals by soaking with *p*-hydroxy mercury benzoate, which reacts covalently with 4-thiouridine at position 8 in tRNA^{fMet}, as proven by standard nucleoside HPLC analysis (Gehrke *et al.*, 1982).

X-ray diffraction data were measured at the electron synchrotron Elettra (Trieste, Italy) at a wavelength of 0.991 Å on a 180 mm MAR Research imaging plate under cryogenic cooling at 123 K. Before mounting, crystals were soaked for 12–24 h in a solution of 100 m*M* HEPES/KOH pH 6.6, 3 m*M* $MgCl_2$, 2 *M* $(NH_4)_2SO_4$ containing 25%(*v*/*v*) glycerol. Diffraction data were processed and scaled with *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997) or with the programs *SCALA* and *AGROVATA* (Collaborative Computational Project, Number 4, 1994).

For Raman measurements, fMet-tRNA^{fMet} was transferred into 20 m*M* HEPES/KOH pH 6.6, 5 m*M* $MgCl_2$ by buffer exchange in Microcon tubes (Amicon) with a 10 kDa exclusion limit and concentrated to 0.8 m*M* (19 mg ml^{-1}). tRNA concentrations were determined from the absorption at 260 nm using an absorption coefficient of 22 ml mg^{-1} cm^{-1}. Home-made cuvettes consisting of cylindrical quartz bodies with quartz bottoms and Teflon stoppers were filled with approximately 10 μl fMet-tRNA^{fMet} solutions. Raman spectra were excited with the 488 nm line of an argon ion laser (Coherent Innova 90). tRNA solutions were measured in the macro chamber with a 90° setup. Single crystals were measured under a microscope (Olympus BH3) in a 180° backscattering geometry. The crystals were positioned in hanging drops of mother liquor. The excitation energy at the sample was about 100 mW for solutions in the

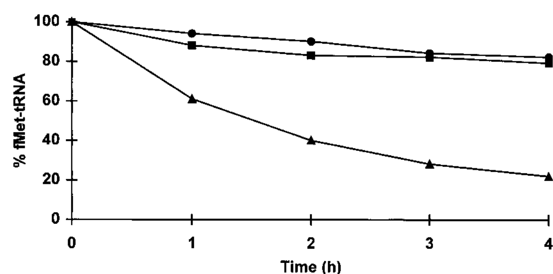

Figure 2
Hydrolysis protection of fMet-tRNA^{fMet} by IF2-C. Complex formation occurred at a concentration of 2.5×10^{-6} *M* (^{14}C)-fMet-tRNA^{fMet} and 5×10^{-5} *M* IF2 or IF2-C. Hydrolysis of the aminoacyl ester linkage of bound IF2 (●), bound IF2-C (■) and free (^{14}C)-fMet-tRNA^{fMet} (▲) was monitored at pH 7.5 and 310 K within the given time intervals. The initial concentration of aminoacylated tRNA was set to 100% and the decrease in (^{14}C)-fMet-tRNA^{fMet}, reflecting the decrease of the complex, was calculated relative to this value.

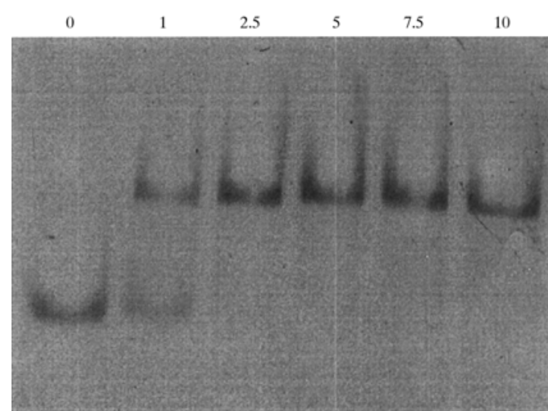

Figure 3
Gel retardation of IF2-C–fMet-tRNA^{fMet}. Autoradiography of a retardation gel showing IF2-C–(^{35}S)-fMet-tRNA^{fMet} complex formation. The lower band represents free (^{35}S)-Met-tRNA^{fMet} and the shifted bands represent (^{35}S)-fMet-tRNA^{fMet} in complex with IF2-C. Molar ratios of IF2-C to fMet-tRNA^{fMet} are given above the lanes. (^{35}S)-fMet-tRNA^{fMet} was used at a concentration of 4×10^{-7} *M* throughout.

macro chamber and about 30 mW for crystals under the microscope. The Raman data were collected with the spectrometer T64000 (Jobin Yvon) at 296 K. Rayleigh scattering was separated with a notch filter. A liquid-nitrogen cooled CCD camera was used as detector.

16 spectral scans of 20 s each were averaged for the region 475–1775 cm^{-1}. Data treatment, including buffer subtraction and background correction, was performed with the software package *Spectramax* (Jobin Yvon) supplemented by several in-house programs. The Raman spectra of crystals were corrected by subtraction of a mother-liquor spectrum normalized to the intense sulfate peak at 980 cm^{-1}. Solution spectra were corrected by subtraction of a buffer spectrum normalized to the buffer band at 1045 cm^{-1}. A slight fluorescence background was then approximated by a polynomial curve.

3. Results

3.1. Complex formation of *B. stearothermophilus* IF2-C and fMet-tRNA^{fMet}

Fig. 1 shows a ribonuclease protection experiment of the ternary complex IF2–GTP–fMet-tRNA^{fMet} compared with the binary complex IF2-C–fMet-tRNA^{fMet}. Both IF2–GTP and IF2-C protect fMet-tRNA^{fMet} from ribonuclease digestion at the same concentrations, whereas the control, free fMet-tRNA^{fMet}, is cleaved by RNase A within 2 min. This indicates that the binding affinity for fMet-tRNA^{fMet} should be in the same order of magnitude for both IF2–GTP and IF2-C.

The rate of deacylation of fMet-tRNA^{fMet} was analyzed free in solution and in complex with IF2 and IF2-C, respectively (Fig. 2). The results clearly indicate hydrolysis protection by IF2-C using the standard hydrolysis-protection conditions (Pingoud *et al.*, 1977) as compared with free fMet-tRNA^{fMet}. The rate of deacylation in the IF2-C–fMet-tRNA^{fMet} complex is similar to that in the complex with the intact IF2, which again suggests that the dissociation constants of both complexes have to be similar. We also investigated the stability of the IF2-C–fMet-tRNA^{fMet} complex by hydrolysis protection at pH values between 5.5 and 6.6 and could observe stability of the aminoacyl ester linkage against hydrolysis for several days under these conditions (not shown), which is important for crystallization.

3.2. Gel-retardation analysis of the IF2-C–fMet-tRNA^{fMet} complex

Complex formation between fMet-tRNA^{fMet} and IF2-C could be directly followed by gel retardation. (^{35}S)-fMet-tRNA^{fMet} was incubated with IF2-C at different ratios. By subsequent gel-shift analysis and autoradiography (Fig. 3) of the gels, a lower band corresponding to fast-moving free (^{35}S)-fMet-tRNA^{fMet} and a shifted band corresponding to the (^{35}S)-fMet-tRNA^{fMet} in complex with IF2-C could be observed. The radioactive label of fMet-tRNA^{fMet} was chosen to be on the attached amino acid in order to detect only free and complexed tRNA which still carries the amino acid and to ensure that the observed dissociation of the complex is not a consequence of deacylation of the tRNA. In Fig. 3, the complex is formed with 4×10^{-7} *M* (^{35}S)-fMet-tRNA^{fMet} and 4×10^{-7} to 4×10^{-6} *M* IF2-C. At a 1:1 molar ratio of both components we observe two bands of equal intensity, the lower band corresponding to free (^{35}S)-fMet-tRNA^{fMet} and the shifted band corresponding to the IF2-C–(^{35}S)-fMet-tRNA^{fMet} complex. With increasing molar excess of IF2-C the lower band disappears. The data establish an upper limit of 2×10^{-7} *M* for the dissociation constant of the complex.

3.3. Crystals of IF2-C–fMet-tRNA^{fMet}

Single crystals were obtained in the presence of 1% polyethylene glycol within 1–3 d by vapour-diffusion equilibration with 100 m*M* HEPES/KOH pH 6.6, 3 m*M* $MgCl_2$, 2 *M* $(NH_4)_2SO_4$ in sitting or hanging drops at a growth temperature of 303 K. Crystal data are summarized in Table 1. Similar crystals have been obtained at 291 K. Using $(NH_4)_2SO_4$ as precipitant may seem surprising at first sight, since a disruption of electrostatic interactions and a loss of complex stability might be expected under high-salt conditions. However, a large number of crystallization studies with elongation factors (*e.g.* Kristensen *et al.*, 1996) and aminoacyl-tRNA synthetases (*e.g.* Eiler *et al.*, 1992; Price *et al.*, 1993; Yaremchuk *et al.*, 1995) have proven that $(NH_4)_2SO_4$ is the precipitant of choice for the crystallization of protein-tRNA complexes. The integrity of IF2-C and the fMet-tRNA recovered from dissolved crystals was proven electrophoretically (not shown). To what extent the tRNA remains aminoacylated in crystals has to be determined by the structure analysis. A potential mercury derivative was obtained by soaking the crystals in 100 m*M* HEPES/KOH pH 6.6, 3 m*M* $MgCl_2$, 2 *M* $(NH_4)_2SO_4$, 25% glycerol as a cryoprotecting solution for data collection at low temperatures and 2.5 m*M* *p*-hydroxy mercury benzoate. This reagent is expected to react covalently with 4-thiouridine, a modified nucleoside located at position 8 in tRNA^{fMet} (Sprinzl *et al.*, 1998). The mercury derivative of the crystal

yielded the best diffraction data collected so far.

At Elettra (Trieste), native IF2-C–fMet-tRNA^{fMet} diffracted to a maximal resolution of 3.1 Å and data with a completeness of 74.8% were collected to 3.8 Å with an $R_{\rm merge}$ of 6.5% ($R_{\rm merge} = \sum |I_{i,j} - \langle I_{i,j}\rangle| / \sum I_{i,j}$, where $I_{i,j}$ are the measurements contributing to the mean reflection intensity $\langle I_i \rangle$). The maximal resolution of the mercury derivative was 3.0 Å and data could be collected to 3.4 Å with a completeness of 83.4% and an $R_{\rm merge}$ of 7.5%.

3.4. Raman analysis of IF2-C–fMet-tRNA^{fMet} crystals

Fig. 4 shows the Raman spectrum of the crystalline IF2-C–fMet-tRNA^{fMet} complex and, for comparison, the spectra of fMet-tRNA^{fMet} and IF2-C in solution. The Raman spectrum of the protein in the region 550–1760 cm^{-1} (Fig. 4*a*) which was published earlier (Misselwitz *et al.*, 1997) is dominated by bands from the amide groups, aliphatic groups and aromatic amino acids. The positions of the amide I and III bands and the C—C stretch band between 903 and 946 cm^{-1} indicate a significant content of both β-sheet and α-helix. The sharp band at 1005 cm^{-1} assigned to phenylalanine provides a convenient identification of protein components in the Raman spectra. The intensity ratio I_{855}/I_{829} of the tyrosine peaks is consistent with a predominant surface localization of phenolic side chains which are hydrogen bonded to solvent H_2O molecules (Peticolas, 1995). The band at 1448 cm^{-1} is assigned to deformation vibrations of CH_2.

The Raman spectrum of fMet-tRNA^{fMet} (Fig. 4*b*) is similar to published tRNA^{fMet} spectra (Thomas & Hartman, 1973). It shows a prominent band at 811 cm^{-1} which has been recognized as a marker of the A-form backbone of RNA (Thomas & Wang, 1988). The band is assigned to a backbone vibration which occurs when the furanose rings are in C3′-*endo*/*anti* conformation. The 1100 cm^{-1} band, assigned to the PO_2^- symmetric stretching vibration, is essentially invariant to backbone conformational changes. The ratio of peak heights of the 811 cm^{-1} band to the band at 1100 cm^{-1} can be used to determine the fraction of the furanose rings with A-type pucker (Peticolas, 1995). Here, a fraction of 84% A-genus conformation is predicted from the observed ratio I_{811}/I_{1100}. The positions of Raman nucleoside marker bands at 669 (rG), 1249 (rC), 1320 (rG) and 1337 cm $^{-1}$ (rA) confirm the A-form conformation (Thomas & Wang, 1988).

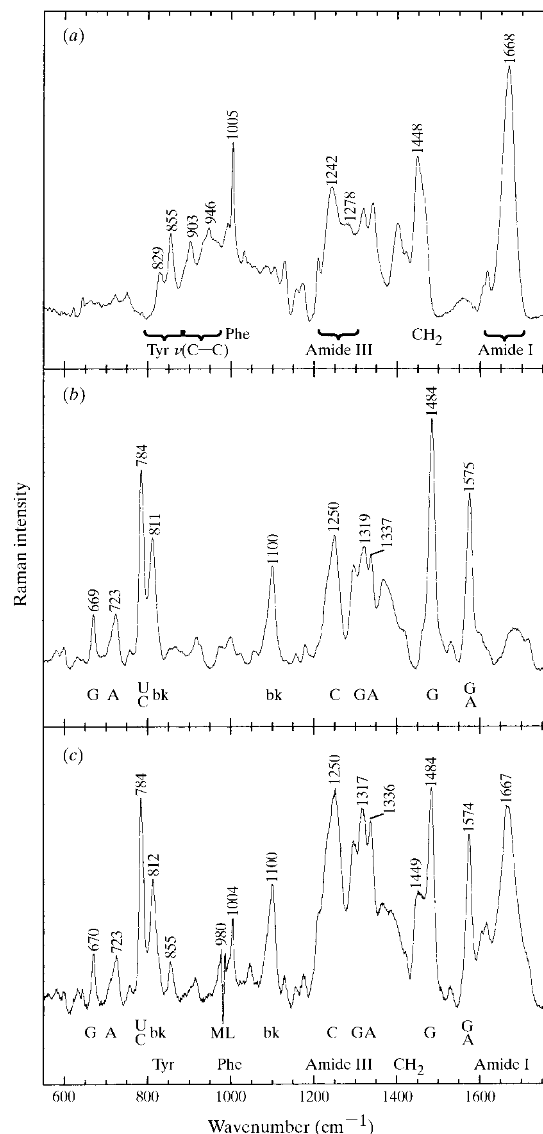

Figure 4

Raman spectra in the region 550–1760 cm^{-1} of (*a*) IF2-C, (*b*) fMet-tRNA^{fMet} and (*c*) the crystalline complex IF2-C–fMet-tRNA^{fMet}. Labels indicate the frequencies in cm^{-1} units and assignments of major bands discussed in the text. The concentrations of IF2 and fMet-tRNA^{fMet} are 1.0 m*M* (24 mg ml^{-1}) and 0.8 m*M* (19 mg ml^{-1}), respectively, in 20 m*M* HEPES/KOH pH 6.6, 5 m*M* $MgCl_2$. Assignments for major peaks were made in accordance with the literature (Thomas & Wang, 1988; Peticolas, 1995). Frequencies are accurate to ±2 cm^{-1}.

The Raman spectrum of the crystalline IF2-C–fMet-tRNA^{fMet} complex (Fig. 4*c*) clearly contains both RNA and protein bands. It is taken as proof that the crystals indeed contain the protein–tRNA complex and not just one of the components. The relative intensities of protein and RNA bands are consistent with equimolar amounts of the components in the crystals. The positions of protein and RNA marker bands coincide within an experimental uncertainty of ±1 cm^{-1}. This suggests that the components are in a native conformation and no major structural changes occur in the crystallized complex. Anisotropy of the single crystals causes intensity differences of some bands depending on their orientation in the laser beam. Therefore, a detailed comparison of the solution spectra of the components and the crystal spectrum of the complex is not feasible.

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