Topography of the *Escherichia coli* Initiation Factor 2/fMet-tRNA$_f^{Met}$ Complex as Studied by Cross-Linking†

Gulnara Yusupova,‡ Joseph Reinbolt,‡ Hiroshi Wakao,§ Soumaya Laalami,||,⊥ Marianne Grunberg-Manago,|| Pascale Romby,‡ Bernard Ehresmann,‡ and Chantal Ehresmann*,‡

Institut de Biologie Moléculaire et Cellulaire, UPR n°9002 du CNRS, 15 rue René Descartes, 67084 Strasbourg Cedex, France, and Institut de Biologie Physico-Chimique, URA n°1139, 13 rue P. et M. Curie, 75005 Paris, France

Received August 16, 1995; Revised Manuscript Received December 8, 1995⊗

ABSTRACT: *trans*-Diamminedichloroplatinum(II) was used to induce reversible cross-links between *Escherichia coli* initiation factor 2 (IF-2) and fMet-tRNA$_f^{Met}$. Two distinct cross-links between IF-2 and the initiator tRNA were produced. Analysis of the cross-linking regions on both RNA and protein moieties reveals that the T arm of the tRNA is in the proximity of a region of the C-terminal domain of IF-2 (residues Asn$_{611}$−Arg$_{645}$). This cross-link is well-correlated with the fact that the C-domain of IF-2 contains the fMet-tRNA binding site and that the cross-linked RNA fragment precisely maps in a region which is protected by IF-2 from chemical modification and enzymatic digestion. Rather unexpectedly, a second cross-link was characterized which involves the anticodon arm of fMet-tRNA$_f^{Met}$ and the N-terminal part of IF-2 (residues Trp$_{215}$−Arg$_{237}$).

Three proteins, transiently associated with ribosomes [initiation factors 1 (IF-1), 2 (IF-2), and 3 (IF-3), and at least one GTP molecule are required to provide the fidelity and efficiency of the translation initiation on natural mRNAs *in vitro* [reviewed in Hartz et al. (1990), Gualerzi and Pon (1990), RajBhandary and Chow (1995), and Grunberg-Manago (1995)]. IF-2 and IF-1 were shown to be essential for cell survival (Laalami et al., 1991; Cummings & Hershey, 1994), and there is evidence that this is also the case for IF-3 (Butler et al., 1986). IF-2, the largest protein involved in translation (97.3 kDa), was shown to display multiple properties [for reviews, see Spurio et al. (1993) and Grunberg-Manago (1995)]. It reduces the dissociation rate constant of all aminoacyl-tRNAs from the mRNA/30S subunit complex, and both IF-2 and IF-3 help the 30S subunit to select the initiator tRNA over other tRNAs (Calogero et al., 1988; Hartz et al., 1989). This selection was shown to be dependent on the formylmethionine charging of the initiator tRNA (Hartz et al., 1989; Varshney et al., 1991; RajBhandary, 1994). IF-2 is endowed with a GTPase activity, which is stimulated by the ribosomal 50S subunit binding. This GTP hydrolysis is probably involved in IF-2 release upon 70S initiation complex formation. The central domain of IF-2 shares structural similarities with the G-domain of the family of guanine nucleotide binding proteins (GTP-binding proteins) (Cenatiempo et al., 1987). In *Escherichia coli*, IF-2 is present under two forms which are expressed from the same gene: IF-2α (97.3 kDa) and IF-2*β* (79.7 kDa) (Plumbridge et al., 1985; Morel-Deville et al., 1990; Sacerdot et al., 1992). IF-2 can be divided into three domains: the N-domain (residues 1−392), a central domain (residues 393−540), and the C-domain (residues 541−890). Several observations suggest that the C-domain contains the tRNA$_f^{Met}$ binding site (Laalami et al., 1991; Gualerzi et al., 1991; Spurio et al., 1993). IF-2 is able to form a binary complex with initiator tRNA which is dependent on its formylation *in vitro* (Sundari et al., 1976; Van der Hofstad et al., 1977; Petersen et al., 1983). In a previous work, we determined the footprint of IF-2 on fMet-tRNA$_f^{Met}$ in the binary IF-2/tRNA complex and in the [30S/IF-2/tRNA/AUG/GTP] complex (Wakao et al., 1989). In both cases, IF-2 shields the T loop and the minor groove of the T stem, suggesting that the interaction between IF-2 and the isolated tRNA or the P-site-bound tRNA is essentially the same.

In the present work, we provide further information on the topography of the IF-2/initiator tRNA complex by using *trans*-diamminedichloroplatinum(II) (*trans*-DDP)[1] to promote coordination bonds between IF-2 and initiator fMet-tRNA$_f^{Met}$. One advantage of this reagent relies on the fact that the coordination bonds between platinum and the potential acceptors on proteins (mainly sulfur atom of cystein and methionine, N of the imidazole ring of histidine) and nucleic acids (N7 of guanine, N1 of adenine, and N3 of cytosine) can be reversed by stronger nucleophilic groups (Tukalo et al., 1987). *trans*-DDP has a square planar geometry where the two reactive chlorines span a 7 Å distance. Two distinct cross-links were identified on both RNA and protein moieties. The analysis revealed that the T arm and the anticodon arm of the tRNA were cross-linked to the C-terminal and to the N-terminal domains of IF-2, respectively. While the former contact was expected from previous data (Wakao et al., 1989), the latter is not.

† This work was supported by grants from the Centre National de la Recherche Scientifique. G.Y. was supported by a short-term fellowship from FEBS and by grants from the French−Russian cooperation.

* To whom correspondence should be sent: C. Ehresmann, Institut de Biologie Moléculaire et Cellulaire, 15 rue René Descartes, 67084 Strasbourg Cedex. Fax: (33) 88.60.22.18. Telephone: (33) 88.40.70.54. E-mail: ehresmc@astorg.U-Strasbg.fr.

‡ UPR n°9002 du CNRS.

§ Present address: Institute of Molecular and Cellular BioSciences, University of Tokyo, Tokyo 113, Japan

|| URA n°1139.

⊥ Present address: URA 1172, Institut de Biologie Moléculaire et d'Ingénierie Génétique, 40 Avenue du Recteur Pineau, 86022 Poitiers Cedex, France.

⊗ Abstract published in *Advance ACS Abstracts,* February 15, 1996.

[1] Abbreviations: *trans*-DDP, *trans*-diamminedichloroplatinum(II).

0006-2960/96/0435-2978$12.00/0

MATERIALS AND METHODS

Chemicals and Enzymes. Folinic acid, *trans*-DDP, and dithioerythreitol (DTE) were purchased from Sigma. Calf intestinal phosphatase, RNase T1, and T4 polynucleotide kinase were from Amersham. RNase kits for oligonucleotide sequencing were from Pharmacia. TPCK-treated trypsin, from Worthington Biochemical Corp., was further purified from contaminating nucleases according to Beeley and Neurath (1968). [γ-^{32}P]ATP, [^{14}C]Met, and [^{14}C]Phe were from Amersham. *E. coli* tRNA$_f^{Met}$, tRNA^{Phe}, and ATP were from Boehringer. The Ultrapac TSK G2000 SW (60 × 7.5 mm) and TSK G4000 SW (30 × 7.5 mm) columns were from LKB. The nucleosil C18 column (125 × 4 mm) was from Macherey-Nagel, and the PVDI-31 column (100 × 4.6 mm) was from Shandon. ProSpin sample preparation cartridges using a ProBlott PVDF membrane attached to an insert in contact with a 3 kDa cutoff filter were purchased from Applied Biosystems. All chemicals used for the Edman's degradation were from Applied Biosystems.

Biochemical Material. IF-2 was purified as described by Mortensen et al. (1991) with some modifications. Acylation of *E. coli* tRNA^{Phe} with [^{14}C]Phe was done according to Wagner and Sprinzl (1980) using phenylalanyl-tRNA synthetase from yeast kindly provided by P. Remy (Strasbourg). NAc[^{14}C]Phe-tRNA^{Phe} was obtained by treatment with acetosuccinimide (Rappoport & Lapidot, 1974) and purified by chromatography on a BD-cellulose column. The *E. coli* tRNA$_f^{Met}$ was labeled at its 5′-end with [γ-^{32}P]ATP and T4 polynucleotide kinase after dephosphorylation with alkaline phosphatase, according to Silberklang et al. (1977). The labeled tRNA$_f^{Met}$ was purified by gel electrophoresis. tRNA$_f^{Met}$ was acylated and formylated as described by Belitzina and Tnalina (1981). The reaction was stopped by phenol extraction, and fMet-tRNA$_f^{Met}$ was precipitated by ethanol and purified by FPLC using a TSK 4000 SW column. The tRNA was then precipitated, washed with 80% ethanol, and dissolved in 5 mM sodium acetate (pH 6.0). The yield of aminoacylation and formylation was estimated as described by Wakao et al. (1989). Routinely, 95% of fMet-tRNA$_f^{Met}$ could be obtained. Before use, the tRNA was renaturated by incubation at 50 °C in the appropriate buffer for 2 min and cooled down slowly (20 min) at room temperature.

Formation of the Cross-Linked Complex between IF-2 and tRNA. An aqueous solution of *trans*-DDP (1 mM) was freshly prepared before each experiment. For platination, IF-2 was incubated with *trans*-DDP at room temperature in the dark in buffer A [5 mM $MgAc_2$, 50 mM NH_4Cl, and 50 mM sodium cacodylate (pH 7.4)]. The concentration of *trans*-DDP and the incubation time were varied as specified in the text. The complex between labeled tRNA and variable concentrations of platinated IF-2 was formed by incubation of IF-2 and tRNA in buffer A for 10 min at 37 °C. The formation of the cross-linked complex was estimated by retention on a nitrocellulose filter (Millipore type HA, 0.45 μm) under either dissociating (buffer A containing 0.45 M NaCl) or nondissociating conditions (buffer A). The cross-linked complex was also analyzed by electrophoresis on 7% polyacrylamide (0.8/30 bis) slab gel in TBE buffer [90 mM Tris-borate (pH 9.0), and 2 mM ethylenediaminetetraacetic acid (EDTA)].

Analysis of the Cross-Linked tRNA Oligonucleotides. The unfractionated reaction mixture was submitted to RNase T1 hydrolysis (0.2 u/μg of tRNA) for 1 h at 37 °C, in the dark. The resulting cross-linked IF-2/oligonucleotide complexes were fractionated from free T1 oligonucleotides by nitrocellulose filtration. The material retained on the filter was washed with 30 mL of buffer A containing 0.45 M NaCl. The nitrocellulose filters were then soaked in 450 μL of 2 M thiourea during 1 h at 37 °C for cross-linking reversal. The supernatant was removed, and the liberated oligonucleotides were precipitated with 3 volumes of ethanol in the presence of 0.3 M sodium acetate (pH 7.0), 5′-labeled and sequenced (see below).

Isolation of the Cross-Linked IF-2/tRNA Complex. For preparative purposes, IF-2 was treated with a 30-fold molar excess of *trans*-DDP for 30 min. The platinated IF-2 (8 nmol) was incubated with 4 nmol of 5′-labeled fMet-tRNA$_f^{Met}$ in a total volume of 2 mL. All reactions were performed in the dark. The cross-linked IF-2/tRNA complex was fractionated from non-cross-linked species by HPLC on a TSK G2000 SW column. Elution was at a flow rate of 0.3 mL/min with 50 mM sodium cacodylate (pH 7.3), 5 mM $MgAc_2$, 0.5 M NaCl, and 6 M urea.

Trypsin Hydrolysis of the Cross-Linked IF-2 and Isolation of the Cross-Linked Peptide/tRNA Complexes. The fractions containing the cross-linked IF-2/tRNA complex were collected and diluted to 0.1 M NaCl and 1.2 M urea by the addition of buffer A. The complex was then digested with RNase-free trypsin at a trypsin/IF-2 ratio of 1/30 (w/w) for 1 h at 37 °C in the dark. The cross-linked peptides were fractionated from non-cross-linked peptides by chromatography on a PVDI-31 anion-exchange HPLC column, equilibrated with 50 mM sodium cacodylate (pH 7.4), 6 M urea, and 0.1 M NaCl. Elution was carried out at a flow rate of 0.3 mL/min with a linear salt gradient from 0.1 to 1 M NaCl in 50 mM sodium cacodylate (pH 7.4) and 6 M urea over 70 min. Fractions were collected every minute and checked for radioactivity. A fraction of the cross-linked peptide/tRNA complexes was diluted 8-fold, loaded on the ProSpin system, and directly submitted to automatic sequencing by the Edman's degradation procedure.

RNase T1 Hydrolysis and Isolation of the Cross-Linked Peptide/Oligonucleotide Complexes. The fractions containing the cross-linked peptide/tRNA complexes (see above) were collected and treated with RNase T1 (3 u/μg) for 45 min at 37 °C in the dark. The cross-linked peptide/oligonucleotide complexes were then fractionated from non-cross-linked oligonucleotides by reverse-phase HPLC chromatography. The sample was diluted 5-fold by 0.05 M NH_4Ac (pH 7.4) and applied on a C18 column equilibrated in solvent A [0.05 M NH_4Ac (pH 7.4)]. Elution was carried out at a flow rate of 0.3 mL/min and as follows: (1) linear gradient from 0 to 30% of solvent B [0.05 M NH_4Ac (pH 7.4) in acetonitrile] for 25 min, (2) 10 min with 30% solvent B, (3) linear gradient from 30 to 50% solvent B for 20 min, (4) linear gradient from 50 to 100% solvent B for 15 min, and (5) 20 min with 100% solvent B.

Sequence Analysis of Cross-Linked Oligonucleotides and Peptides. Each peak was collected and vacuum dried on a SpeedVac concentrator. After they were dissolved in 100 μL of 0.05 M DTE, the samples were incubated for 30 min at 37 °C for cross-linking reversal. Each sample containing the liberated oligonucleotides and peptides was divided into two fractions. One aliquot was submitted to automated

Edman's degradation, using an Applied Biosystems 470A protein sequencer equipped with a PTH 120 A analyzer. The other one was submitted to 5′-end labeling as described above. The resulting 5′-end-labeled oligonucleotides were fractionated by electrophoresis on a 20% polyacrylamide (1/20 bis)/8 M urea slab gel. After autoradiography, the fragments were excised, eluted according to Maxam and Gilbert (1977), and repurified by a second electrophoresis on a 20% polyacrylamide/8 M urea gel. After elution, the RNA was precipitated with ethanol in the presence of 10 μg of tRNA as carrier, dissolved in 10 μL of water, incubated for 2 min at 90 °C, and sequenced according to Donnis-Keller et al. (1977). Digestion was with RNase T1 (0.005 u/μg of RNA), RNase U2 (0.2−0.5 u/μg of RNA), RNase PhyM (1 u/μg of RNA), and *Bacillus cereus* RNase (1 u/μg of RNA). Incubation was at 55 °C for 10 min in sodium citrate (33 mM, pH 5.0) containing 1 mM EDTA, in the presence of 8 M urea for RNase T1 and PhyM, and in the absence of urea for *B. cereus* RNase, and in sodium citrate (20 mM, pH 3.5) containing 1 mM EDTA in the presence of 8 M urea for RNase U2. The latter was obtained by incubation of RNA in 6 μL of 50 mM sodium carbonate (pH 8.9) at 90 °C for 15 or 30 min. Analysis of the digests was carried out by electrophoresis on a 20% polyacrylamide/8 M urea slab gel.

RESULTS

Conditions and Specificity of Cross-Linking. The overall strategy for identification of the cross-linked oligonucleotide/peptide complexes is summarized in Figure 1. The binding of IF-2 pretreated with *trans*-DDP to fMet-tRNA$_f^{Met}$ was verified by nitrocellulose filtration assays (Figure 2). Increasing concentrations of platinated IF-2 were incubated with labeled fMet-tRNA$_f^{Met}$, and the incubation mixture was filtered in the presence or in the absence of 0.45 M NaCl (dissociating or nondissociating conditions, respectively) (Figure 2). The retention is reduced in the presence of NaCl, most likely due to the dissociation of the non-cross-linked complexes during filtration. In the absence of *trans*-DDP, the retention is strongly decreased, due to the low stability of the binary complex, in agreement with previous data (Majumbar et al., 1976; Sundari et al., 1976). The specificity of the cross-linking reaction was checked by following cross-linking of platinated IF-2 with NAcPhe-tRNA^{Phe}, a noncognate substrate. The results show that no significant cross-link was observed, even in the presence of a 20-fold molar excess of tRNA, in the presence or in the absence of NaCl.

In order to minimize the formation of intramolecular RNA cross-links, IF-2 was platinated prior the formation of the IF-2/tRNA complex. The platinated IF-2 was preincubated with increasing concentrations of *trans*-DDP and allowed to bind labeled fMet-tRNA$_f^{Met}$. The resulting complexes were then analyzed by gel electrophoresis in nondenaturing conditions (Figure 3). At low *trans*-DDP concentrations, the labeled tRNA is shifted to a single band which migrates as the non-cross-linked IF-2/fMet-tRNA complex, indicating that the stoichiometry of the complex is unaffected. The yield of the binary IF-2/fMet-tRNA complex is significantly increased upon increasing platination. However, at higher *trans*-DDP concentrations, additional slowly migrating bands are observed which probably correspond to intermolecular IF-2 cross-links. Aggregates, which do not enter the gel, are observed at the highest *trans*-DDP concentrations.

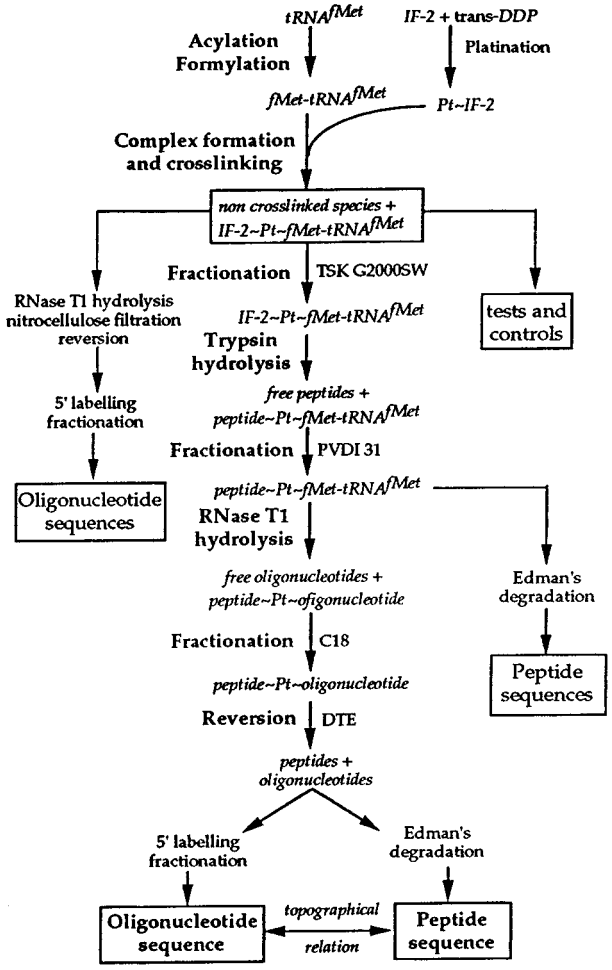

FIGURE 1: Strategy used to characterize the cross-linked oligonucleotide/peptide complexes from fMet-tRNA$_f^{Met}$-platinated IF-2.

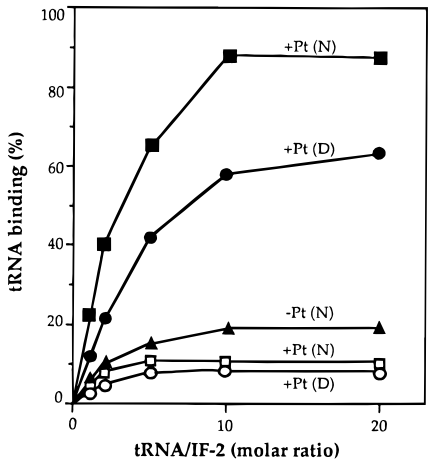

FIGURE 2: Binding of IF-2 to fMet-tRNA$_f^{Met}$ (black symbols) or NAcPhe-tRNA^{Phe} (open symbols) as measured by nitrocellulose membrane filtration. The experiments were conducted with platinated IF-2 (+Pt) in the presence (D) or in the absence (N) of 0.45 M NaCl.

Therefore, in the following experiments, platination was conducted at a *trans*-DDP/IF-2 ratio of 30/1, for 30 min.

Identification of the Regions of fMet-tRNA$_f^{Met}$ Cross-Linked to IF-2. Cross-linked IF-2/oligonucleotides generated by RNase T1 digestion of the tRNA-platinated IF-2 complex were fractionated from free oligonucleotides by nitrocellulose filtration (Figure 1). The retained oligonucleotides were

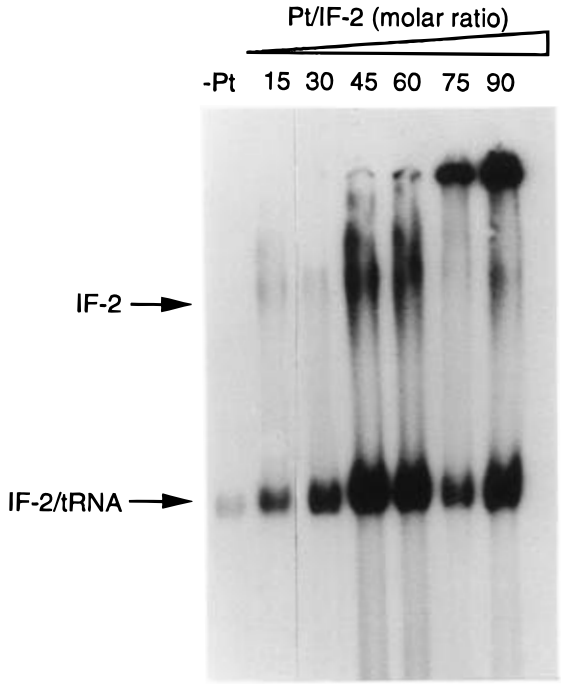

FIGURE 3: Electrophoretic separation of the cross-linked fMet-tRNA$_f^{Met}$/IF-2 complex on a 7% polyacrylamide gel under native conditions. The −Pt lane is non-cross-linked [^{32}P]fMet-tRNA$_f^{Met}$/IF-2 complex. The Pt/IF-2 lanes are cross-linked [^{32}P]fMet-tRNA$_f^{Met}$/platinated IF-2 complex in the presence of an increasing amount of *trans*-DDP.

labeled and sequenced after cross-linking reversal. A control experiment shows that oligonucleotides are only retained when IF-2 is platinated (Figure 4A). Furthermore, no nucleotidic material could be retained when a RNase T1 digest of fMet-tRNA$_f^{Met}$ is filtered in the presence of platinated IF-2. Therefore, RNA fragments isolated from this experimental protocol are specifically cross-linked to IF-2.

From a total of six independent experiments, it became evident that all subfragments arise from two distinct tRNA$_f^{Met}$ regions (Figure 4B). A first group of fragments covers the 3′-half of the tRNA and overlaps the T arm (nucleotides 46−76). Fragments from the second group cover the anticodon arm (nucleotides 23−45). No cross-linked fragments have been isolated from other regions of the tRNA molecule. In the experiment shown in figure 4, the material contained in bands 1−11 was clearly identified. However, fragments shorter than these two fragments were also observed (Figure 4A). Some of them could not be unambiguously identified due to their very short length, but most of them appear to arise from nonenzymatic cleavage of the anticodon loop. This type of cleavage is frequently observed in RNAs and is assumed to reflect the intrinsic fragility of the ribose−phosphate backbone (Dock-Brégeon et al., 1987). Since nonenzymatic cuts might have been produced after reversion of the cross-links, such fragments have to be taken into consideration. The two shortest identified RNase T1 fragments ($T_{54}-G_{63}$ and $Cm_{32}-G_{42}$) should therefore contain cross-linking sites. It is worthy of note that the same RNA regions are found cross-linked with IF-2β, the shortest form of IF-2, which is expressed from the same gene (results not shown).

Isolation and Characterization of IF-2 Peptides Cross-Linked to fMet-tRNA. The strategy devised to identify the cross-linked peptides is summarized in Figure 1. The complex between labeled fMet-tRNA and platinated IF-2 was formed at high scale, and around 30% of the tRNA was found cross-linked to IF-2. The cross-linked complex was purified by gel filtration (Figure 5) which allows removal of the free tRNA and most of the non-cross-linked IF-2, as well as some minor species probably resulting from intermolecular IF-2 cross-linking. The collected fraction gives one homogeneous band when analyzed by polyacrylamide gel electrophoresis (inset of Figure 5). After trypsin digestion of the purified cross-linked complex, the resulting tRNA-bound peptides were fractionated from the non-cross-linked IF-2 peptides by anion-exchange chromatography on a PVDI column under denaturing conditions (not shown). Two radioactive peaks were obtained: a minor one (∼20%) eluted at a high salt concentration (1 M NaCl) and a major one eluted at a lower salt concentration (0.7−0.8 M NaCl). Peptides were only found in the major peak. Since tryptic peptides arising from trypsin digestion of IF-2 are not retained on the column (not shown), the peptides present in the major peak do correspond to the cross-linked peptides. The peptide content of the major peak was analyzed by automatic sequencing by the Edman's degradation procedure, and the presence of two defined peptides in approximately equimolar amount was revealed. Since the sequence of IF-2 is known, they could be unambiguously identified. They correspond to amino acid residues $Trp_{215}-Arg_{237}$ (peptide A) and $Asn_{611}-Arg_{645}$ (peptide B) from the N- and C-domains, respectively. No other peptidic material could be detected in several independent experiments. In some experiments, the N-terminal peptide was more extended, covering amino acid residues from $Met_{209}-Arg_{237}$.

Correlation between Cross-Linked Oligonucleotides and Peptides. The fraction containing the tRNA/peptide species isolated from the PVDI column was submitted to T1 RNase digestion. The resulting coordinated oligonucleotide/peptide complexes were fractionated from non-cross-linked oligonucleotides by HPLC on a C18 reverse-phase column (Figure 6). Each fraction was submitted to DTE treatment (for cross-linking reversal) and divided into two fractions. Both fractions were analyzed for their peptide and RNA content, respectively. In the latter case, the eventual nucleotidic material was 5′-labeled and fractionated by gel electrophoresis (Figure 6). The labeled bands were excised, eluted, and sequenced. Examples of sequence gels are shown in Figure 7, and the results are summarized in Figure 8.

Two major peaks (2 and 3) contain peptides B and A, respectively. The complete sequences of peptide A ($Trp_{215}-Arg_{237}$) and peptide B ($Asn_{611}-Arg_{645}$) were unambiguously determined. In some experiments, a minor peak was detected corresponding to a peptide containing residues $Glu_{370}-Arg_{381}$, which escaped detection during the sequencing analysis of the preceding step (PVDI fractionation), presumably due to its low amount. However, the absence of detectable RNA associated to this peak suggests that this peptide should have coeluted with tRNA-bound peptides during PVDI chromatography.

As a result of five independent experiments, it turns out that peak 2 contains fragments derived from the T arm. In the experiment of Figure 7, one RNA fragment covering $m^7G_{46}-G_{70}$ was identified. Shorter fragments were also isolated in other experiments; the shortest one extends from $m^7G_{46}-G_{63}$. Therefore, peptide B (from the C-domain of IF-2) is cross-linked to the T arm of fMet-tRNA. The

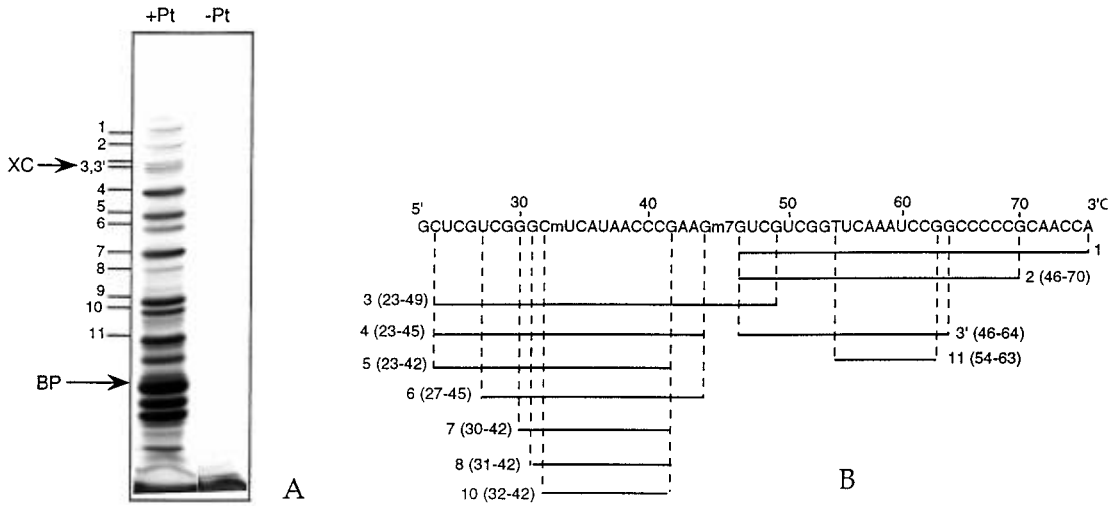

FIGURE 4: (A) Fractionation of the cross-linked oligonucleotides on a 20% polyacrylamide−urea gel electrophoresis. The cross-linked IF-2/oligonucleotide complexes were subjected to 5′-end labeling and to reversion of the cross-links before fractionation. +Pt is the cross-linking experiment as described in Materials and Methods; −Pt is the control experiment in the absence of *trans*-DDP. XC and BP represent xylen cyanol and bromophenol blue positions, respectively. (B) Diagram of fMet-tRNA$_f^{Met}$ fragments found to be cross-linked to IF-2. tRNA fragments are numbered as in panel A. Fragment 9 (C_{51}−G_{63}) arises from a nonenzymatic cut and therefore was not considered.

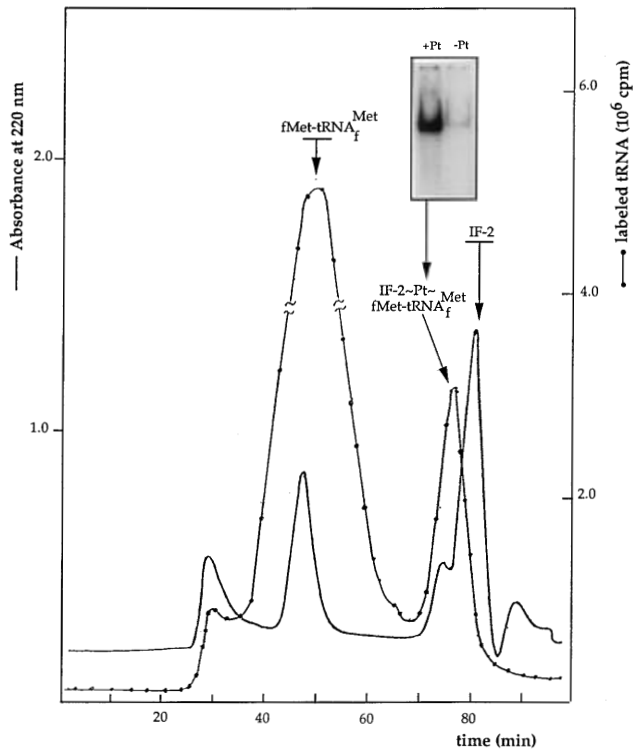

FIGURE 5: Purification of the cross-linked platinated IF-2/fMet-tRNA$_f^{Met}$ complex by chromatography on a TSK G2000 SW column under dissociation conditions as described in Materials and Methods. An electrophoregram of the isolated cross-linked complex (+Pt) and of the native non-cross-linked complex as a control (−Pt) is shown in the inset.

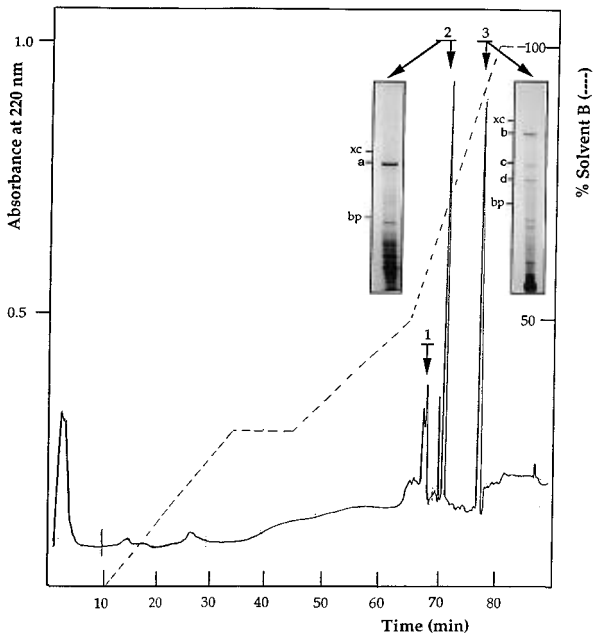

FIGURE 6: Separation of IF-2 peptides generated by trypsin digestion cross-linked to RNase T1 oligonucleotides of fMet-tRNA$_f^{Met}$ by reverse phase HPLC: (—) absorbance at 220 nm (left scale); (- - -) acetonitrile concentration (right scale). The flow rate is 0.3 mL/min. Experimental details are given in Materials and Methods. Radioactive cross-linked oligonucleotides present in peaks 2 and 3 are shown in the insets. After sequencing, the bands have been assigned as follows: a, m^{7}G$_{46}$−G$_{70}$; b, C$_{23}$−G$_{45}$; c, U$_{27}$−G$_{42}$; d, U$_{33}$−G$_{42}$.

analysis of peak 3, containing peptide A (from the N-domain of IF-2), shows the presence of oligonucleotides derived from the anticodon arm of fMet-tRNA. The length of these fragments varies between C_{23}−G_{45} and C_{32}−G_{42} (see Figure 6). It has to be noted that, in the experimental conditions used, free oligonucleotides are not retained on the column.

DISCUSSION

The present study shows that *trans*-DDP induces cross-links between the T arm of fMet-tRNA$_f^{Met}$ and a region (Asn_{611}−Arg_{645}) from the C-domain of IF-2 and between the anticodon arm and Trp_{215}−Arg_{237} from the N-domain. The different controls indicate that the isolated material corresponds to the cross-linked oligonucleotide/peptide complexes and does not result from nonspecific interactions. However, it is difficult to identify with precision the cross-linked residues, since there are several potential candidates according to the known reaction specificity of *trans*-DDP. Also, the occurrence of several cross-links in each domain cannot be ruled out. These cross-links reflect topographical vicinity in a range of 7 Å. Noteworthy, is the fact that the same RNA regions were found cross-linked with IF-2β, the shortest

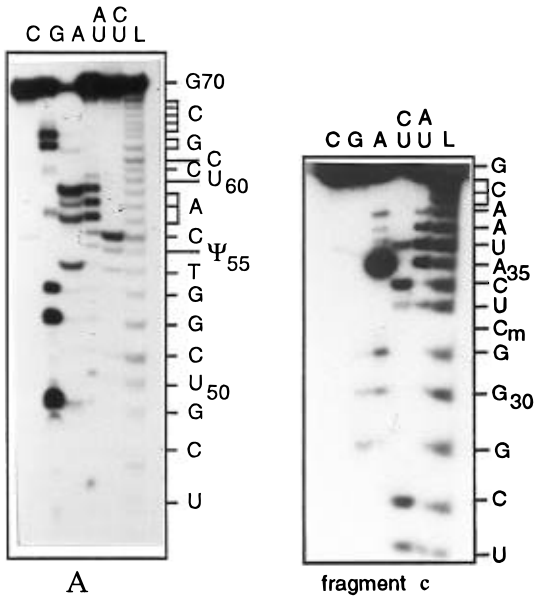

FIGURE 7: Sequence analysis of two cross-linked tRNA$_f^{Met}$ fragments found cross-linked to peptides A and B. Sequence of fragments a ($m^7G_{46}-G_{70}$) and c ($U_{27}-G_{42}$) from Figure 6. Lanes: (C) control in the absence of enzyme; (G,A, AU, and CU) hydrolysis with RNases T1, U2, and PhyM and RNase from *B. cereus*, respectively; (L) alkaline ladder. Conditions of the digestions are given in Materials and Methods.

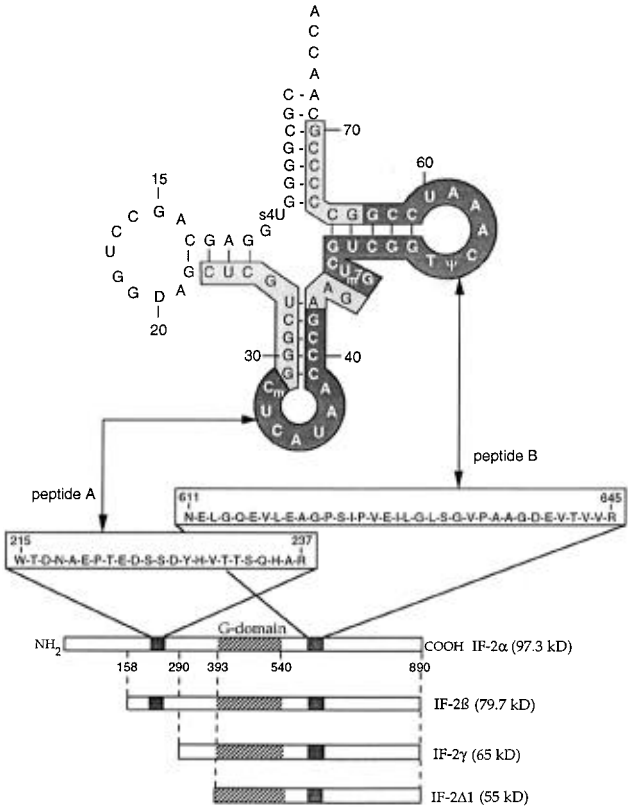

FIGURE 8: Schematic representation of initiator tRNA$_f^{Met}$ and of *E. coli* IF-2. RNase T1 fragments of fMet-tRNA$_f^{Met}$ reproducibly found cross-linked to peptides A and B of IF-2 are shown in light gray, and the shortest ones are indicated in dark gray. The sequences of the two corresponding cross-linked peptides are boxed. Different *E. coli* IF-2 (α and β) and truncated active IF-2 fragments are represented [from Sacerdot et al. (1992) and Laalami et al. (1991)].

form of IF-2 expressed from the same gene, indicating that fMet-tRNA is recognized in a similar way by both forms of IF-2. This result is not surprising since it was shown that each form of IF-2 is sufficient to allow maximal growth of *E. coli* when supplied in excess (Sacerdot et al., 1992).

The former cross-linked region ($Asn_{611}-Arg_{645}$) precisely maps in a region which was found to be protected from chemical modification and enzymatic digestion by IF-2 (Petersen et al., 1981; Wakao et al., 1989). On the other hand, the fact that fMet-tRNA is cross-linked to the C-domain of IF-2 is in agreement with other observations that suggest that the C-domain contains the fMet-tRNA binding site. Indeed, it was shown that a 55 kDa truncated form of *E. coli* IF-2 lacking the N-domain can sustain bacterial growth when supplied in excess (Laalami et al., 1991), while the isolated G-domain was not able to support growth (Laalami et al., 1994). Previous experiments showed that IF-2γ, a proteolytic 65 kDa fragment of IF-2 (lacking the first 289 amino acids), still retains a half-reduced activity in dipeptide synthesis, as compared to the entire IF-2 (Cenatiempo et al., 1987). The C-domain of *Bacillus stearothermophilus* IF-2 was also found to specifically bind fMet-tRNA and to protect it from alkaline pH treatment, albeit with a 5−10-fold reduced efficiency, as compared to that of the complete molecule (Gualerzi et al., 1991). Two mutations that affect tRNA binding were located in a conserved region of the C-domain (Spurio et al., 1993). Furthermore, the C-domain of *B. stearothermophilus* IF-2 was found to provide a weak stimulation of fMet-tRNA to poly(AUG)-programmed ribosomes, despite its lack of affinity to the ribosomes. However, no stimulation was observed in response to natural mRNAs, and the fMet-tRNA bound in the presence of the C-domain to poly(AUG)-programmed 70S ribosomes was not reactive to puromycine (Spurio et al., 1993). These observations confirm the role of the C-domain in fMet-tRNA binding but underline a requirement for native IF-2 for optimal binding of fMet-tRNA in response to natural mRNAs and to its correct placement in the ribosomal P-site.

The unexpected finding that the anticodon arm can be cross-linked to amino acid residues $Trp_{215}-Arg_{237}$ in the N-domain of IF-2α might reflect a second interacting region. If this interaction exists, it would be rather unstable and/or dynamic so that no protection could be detected. However, enhanced spontaneous cleavages were observed in the anticodon loop in the presence of IF-2, as well as an increased reactivity of G_{42} at position N7 (Wakao et al., 1989). This was interpreted as an increased flexibility of the anticodon arm, as a result of IF-2 binding. These findings are possibly related with the observation that the N-domain of IF-2 appears to be required for optimal functions of the factor. It was further shown that fMet-tRNA protects several regions of *B. stearothermophilus* IF-2 from trypsinolysis (Severini et al., 1992) mainly located in the GTP binding domain and to a lesser extent between the N-terminal domain and G-domain. These authors postulate that, upon fMet-tRNA$_f^{Met}$ binding, long-distance tertiary interactions between IF-2 domains may take place. Therefore, it can be assumed that the C-domain of IF-2 primarily interacts with the T arm of fMet-tRNA, while the N-domain might take part in some conformational adjustment of the anticodon arm. This would allow the tRNA to acquire a more favorable conformation of the anticodon loop favoring the adjustment of the initiator tRNA in the P-site.

Our previous work suggested that IF-2 covers the top of the amino acid arm of the L-shaped tRNA molecule near

the elbow, sitting in the minor groove (Wakao et al., 1989). The present cross-linking data imply that this interaction is most likely mediated by the C-domain. The second implication of this work is that the region encompassing residues 215−237 is able to come near the anticodon arm. Secondary structure prediction suggests that these residues are in a very flexible region connecting the N-terminal domain, which contains highly charged and unusual repeating structures (residues 104−155 and 167−214), while the rest of the molecule appears to be highly organized (Sacerdot et al., 1984; Cenatiempo et al., 1987). This flexibility might allow cross-linking between these two regions. The functional significance of a possible contact between the anticodon arm and the N-domain of IF-2 still remains to be investigated in more detail.

ACKNOWLEDGMENT

We are grateful to H. Moine for helpful discussions and to C. Lichté for skillful technical assistance.

REFERENCES

Beeley, J. G., & Neurath, H. (1968) *Biochemistry 7*, 1239−1251.
Belitzina, N. V., & Tnalina (Yusupova), G. Zh. (1981) *Mol. Biol. 15*, 1234−1244.
Butler, J. S., Springer, M., Dondon, J., Graffe, M., & Grunberg-Manago, M. (1986) *J. Mol. Biol. 192*, 767−780.
Calogero, R. A., Pon, C. L., Canonaco, M. A., & Gualerzi, C. O. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 6427−6431.
Cenatiempo, Y., Deville, F., Dondon, J., Grunberg-Manago, M., Sacerdot, C., Hershey, J. W. B., Hansen, H. F., Petersen, H. U., Clark, B. F. C., Kjeldgaard, M., la Cour, T. F. M., Mortensen, K. K., & Nyborg, J. (1987) *Biochemistry 26*, 5070−5076.
Cummings, H. S., & Hershey, J. W. (1994) *J. Bacteriol. 176*, 198−205.
Dock-Brégeon, C., & Moras, D. (1987) *Cold Spring Harbor Symposium on Quantitative Biology*, Vol. L11, pp 113−121, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Donnis-Keller, H., Maxam, A. M., & Gilbert, W. (1977) *Nucleic Acids Res. 4*, 2527−2538.
Grunberg-Manago, M. (1995) *Escherichia coli and Salmonella typhymurium: cellular and molecular biology* (Neidhart, F. C., Ingraham, J. L., Brookes Low, H., Magasanik, B., Schachter, M., & Umbarger, H. E., Eds.) American Society for Microbiology, Washington D.C.
Gualerzi, C., & Pon, C. (1990) *Biochemistry 29*, 5881−5889.
Gualerzi, C., Severini, M., Spurio, R., La Teana, A., & Pon, C. (1991) *J. Biol. Chem. 266*, 16356−16362.
Hartz, D., McPheeters, D. S., & Gold, L. (1989) *Genes Dev. 3*, 1899−1912.
Hartz, D., Binkley, J., Hollinsworth, T., & Gold, L. (1990) *Genes Dev. 4*, 1790−1800.
Laalami, S., Putzer, H., Plumbridge, J. A., & Grunberg-Manago, M. (1991) *J. Mol. Biol. 220*, 335−349.
Laalami, S., Timofeev, A. V., Putzer, H., Leautey, Y. J., & Grunberg-Manago, M. (1994) *Mol. Microbiol. 11*, 293−302.
Majumdar, A., Bose, K. K., Gupta, N. K., & Wahba, A. J. (1976) *J. Biol. Chem. 251*, 137−140.
Maxam, A. M., & Gilbert, W. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 560−564.
Morel-Deville, F., Vachon, G., Sacerdot, C., Cozzone, A. J., Grunberg-Manago, M., & Cenatiempo, Y. (1990) *Eur. J. Biochem. 188*, 605−614.
Mortensen, K. K., Nyengaard, N. R., Hershey, J. W. B., Laalami, S., & Sperling-Petersen, H. U. (1991) *Biochimie 73*, 983−989.
Petersen, H. U., Kruse, T. A., Worm-Leonhard, H., Siboska, G. E., Clark, B. F. C., Boutorin, A. S., Remy, P., Ebel, J.-P., Dondon, J., & Grunberg-Manago, M. (1981) *FEBS Lett. 128*, 161−165.
Petersen, H. U., Wikman, F. P., Siboska, G. E., Worm-Leonhard, H., & Clark, B. F. C. (1983) in *Gene expression* (Clark, B. F. C., & Petersen, H. U., Eds.) pp 41−57, Alfred Benzon Symposium 19, Munksgaard, Copenhagen.
Plumbridge, J. A., Deville, F., Sacerdot, C., Petersen, H. U., Cenatiempo, Y., Cozzone, A., Grunberg-Manago, M., & Hershey, J. W. B. (1985) *EMBO J. 4*, 223−229.
RajBhandary, U. L. (1994) *J. Bacteriol. 176*, 547−552.
RajBhandary, U. L., & Chow, C. M. (1995) in *tRNA: Structure, Biosynthesis, and Function* (Söll, D., & RajBhandary, U. L., Eds.) pp 511−528, American Society for Microbiology, Washington D.C.
Rappoport, S., & Lapidot, Y. (1974) *Methods Enzymol. 29*, 685−688.
Sacerdot, C., Dessen, P., Hershey, J. W. B., Plumbridge, J. A., & Grunberg-Manago, M. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 7787−7790.
Sacerdot, C., Vachon, G., Laalami, S., Morel-Deville, F., Cenatiempo, Y., & Grunberg-Manago, M. (1992) *J. Mol. Biol. 225*, 67−80.
Severini, M., Choli, T., La Teana, A., & Gualerzi, C. (1992) *FEBS Lett. 297*, 226−228.
Silberklang, M., Gillum, A. M., & RajBhandary, U. C. (1977) *Nucleic Acids Res. 4*, 4091−4108.
Spurio, R., Severini, M., La Teana, A., Canonaco, M. A., Pawlik, R. T., Gualerzi, C., & Pon, C. L. (1993) in *The Translational Apparatus, Structure, Function, Regulation, Evolution* (Nierhaus, K. H., et al., Eds.) pp 241−252, Plenum Press, New York and London.
Sundari, R. M., Stringer, E. A., Schulmann, L. H., & Maitra, U. (1976) *J. Biol. Chem. 251*, 3338−3345.
Tukalo, M., Kubler, M.-D., Kern, D., Mougel, M., Ehresmann, C., Ebel, J.-P., Ehresmann, B., & Ehresmann, C. (1987) *Biochemistry 26*, 5200−5208.
Van der Hofstad, G. A. J. M., Foekens, J. A., Bosch, L., & Voorma, H. O. (1977) *Eur. J. Biochem. 77*, 69−75.
Varshney, U., Lee, C. P., Seong, B. L., & RajBhandary, U. L. (1991) *J. Biol. Chem. 266*, 18018−18024.
Wagner, J., & Sprinzl, M. (1980) *Eur. J. Biochem. 108*, 213−221.
Wakao, H., Romby, P., Westhof, E., Laalami, S., Grunberg-Manago, M., Ebel, J.-P., Ehresmann, C., & Ehresmann, B. (1989) *J. Biol. Chem. 264*, 20363−20371.

BI9519415