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Initiation factor IF 2 binds to the α-sarcin loop and helix 89 of *Escherichia coli* 23S ribosomal RNA

ANNA LA TEANA,[1] CLAUDIO O. GUALERZI,[2] and ALBERT E. DAHLBERG[3]

[1]Insitute of Biochemistry, University of Ancona, 60131 Ancona, Italy
[2]Laboratory of Genetics, Department of Biology MCA, University of Camerino, 62032 Camerino (MC), Italy
[3]J.W. Wilson Laboratory, Department of Molecular & Cell Biology and Biochemistry, Brown University, Providence, Rhode Island 02912, USA

ABSTRACT

During initiation of protein synthesis in bacteria, translation initiation factor IF2 is responsible for the recognition of the initiator tRNA (fMet-tRNA). To perform this function, IF2 binds to the ribosome interacting with both 30S and 50S ribosomal subunits. Here we report the topographical localization of translation initiation factor IF2 on the 70S ribosome determined by base-specific chemical probing. Our results indicate that IF2 specifically protects from chemical modification two sites in domain V of 23S rRNA, namely A2476 and A2478, and residues around position 2660 in domain VI, the so-called sarcin-ricin loop. These footprints are generated by IF2 regardless of the presence of fMet-tRNA, GTP, mRNA, and IF1. IF2 causes no specific protection of 16S rRNA. We observe a decreased reactivity of residues A1418 and A1483, which is an indication that the initiation factor has a tightening effect on the association of ribosomal subunits. This result, confirmed by sucrose density gradient analysis, seems to be a universally conserved property of IF2.

Keywords: ribosomal topography; rRNA function; subunit association; translation initiation

INTRODUCTION

Three protein factors (initiation factors IF1, IF2, and IF3) are required for the efficiency and fidelity of translation initiation in bacteria. IF2 is the largest of the three factors (97.3 kDa in *Escherichia coli*) and belongs to the same GTPase subfamily as EF-Tu and EF-G (Bourne et al., 1991). This factor specifically recognizes the acceptor arm of fMet-tRNA (Guenneugues et al., 2000), which contains a blocked αNH_2 group, and promotes the binding of initiator tRNA at the ribosomal P-site (for review, see Gualerzi & Pon, 1990).

The function and structure of IF2 have been the object of extensive studies for many years, but the mechanism of its binding to and release from the ribosome and its topographical localization are still obscure. Protein–protein crosslinking experiments have identified ribosomal proteins S13 (Heimark et al., 1976) and L7/L12 (Boileau et al., 1983) as IF2 neighbors on the 30S and 50S subunits, respectively. Based on the finding that all translational GTPases interact with protein L7/L12, it has been proposed that elongation factors EF-Tu and EF-G, initiation factor IF2, and termination factor RF3 bind to overlapping sites on the ribosome (e.g., see Brock et al., 1998, and references therein; Tomsic et al., 2000, and references therein). This overlap has been clearly demonstrated for the elongation factors by chemical probing with base-specific reagents (Moazed et al., 1988). In fact, EF-Tu, bound to ribosomes in a ternary complex with aminoacyl-tRNA and GTP in the presence of kirromycin, was found to protect the α-sarcin region of 23S rRNA strongly shielding G2655 and G2661 and, more weakly, A2660 and A2665. EF-G, on the other hand, was found to protect strongly A2660, G2655, and G2661 in a ribosomal complex stabilized by fusidic acid or by GTP analogs. In addition, EF-G was found to protect the so-called thiostrepton region around position 1067 (Moazed et al., 1988).

Until now, attempts to identify the rRNA regions in contact with IF2 have not been particularly successful. When the interaction between IF2 and the 30S ribo-

Reprint requests to: Albert E. Dahlberg, Department of Molecular & Cell Biology and Biochemistry, Brown University, 69 Brown Street, Providence, Rhode Island 02912, USA; e-mail: Albert_Dahlberg@Brown.edu.

Abbreviations: DMS: dimethylsulfate; CMCT: 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluene sulphonate; DTT: dithiothreitol.

somal subunit was investigated by phosphate alkylation with ethylnitrosourea, a large number of 16S rRNA bases distributed throughout the particle were found to be either protected or more exposed in the presence of the factor, leading to inconclusive results (Wakao et al., 1990). More recent attempts to identify the IF2 binding site using base-specific chemical probes have also been unsuccessful (Moazed et al., 1995).

Information about the topographical localization of IF2 on the ribosome is necessary to understand the mechanism of action of this factor and, in particular, its GTPase activity and recycling. To identify the location of IF2 on the ribosome, we have taken advantage of the functional interchangeability between *E. coli* and *Bacillus stearothermophilus* IF2 (Brombach et al., 1986) and the stronger affinity displayed by the thermophilic factor for the *E. coli* ribosome (e.g., La Teana et al., 1996). Here we present evidence that IF2 can interact with the 23S rRNA and that its binding site partially overlaps that of elongation factors EF-Tu and EF-G. Chemical probing of the 16S rRNA and sucrose density gradient analysis support earlier reports (Grunberg-Manago et al., 1975; Pestova et al., 2000) that IF2 enhances the association between ribosomal subunits.

RESULTS

IF2 binding to 23S rRNA in 70S ribosomes

During initiation of protein synthesis, IF2 binds to both ribosomal subunits. It has been assumed but not demonstrated that the localization of IF2 on the ribosome overlaps that of EF-Tu and EF-G. To obtain evidence of a direct interaction between IF2 and ribosomal RNA, complexes were formed between *B. stearothermophilus* IF2 and *E. coli* 30S or 70S ribosomes in the presence or absence of fMet-tRNA, GTP, poly(AUG), and IF1. These complexes were then treated with base-modifying reagents; DMS to probe A at N1, C at N3, and G at N7, kethoxal to probe G at N1 and N2, and 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluene sulphonate (CMCT) to probe U at N3 and G at N1 (Merryman & Noller, 1998). The results demonstrate that in "loose couple" 70S ribosomes (see Materials and Methods), IF2 protects two regions within the 23S rRNA, namely the loop around position 2660 (Fig. 1A) and nt 2476 and 2478 (Fig. 1B). The same effects of IF2 on 23S rRNA modification were observed in IF2-70S binary complexes (Fig. 1) and in complete initiation complexes formed in the presence of IF1 and GTP and containing both fMet-tRNA and poly(AUG) (data not shown). The rRNA was extracted from these complexes, and in all cases it displayed the typical pattern of P-site protections for both 16S and 23S rRNA, confirming that fMet-tRNA was correctly placed in the P-site in these initiation complexes.

The loop around position 2660 corresponds to the so-called sarcin-ricin domain (SRD; Wool et al., 2000, and references therein), a universally conserved region and the target of two toxins that abolish protein synthesis, namely α-sarcin, which cleaves the G2661-A2662 phosphodiester bond, and ricin, which removes A2660 by an N-glycosidase reaction. Within the SRD, which also constitutes a binding site for elongation factors EF-Tu and EF-G (Wool et al., 2000), IF2 protects residues G2655 and G2661 from kethoxal modification and A2665 from DMS modification while enhancing the reactivity of A2660 towards DMS (Fig. 1A). This pattern obtained with IF2 is similar but not identical to that observed with EF-Tu and EF-G (Moazed et al., 1988).

The second site of IF2-50S interaction is in the loop at the end of helix 89, which emerges from the central loop of domain V. Both A2476 and A2478 are protected by IF2 from DMS modification (Fig. 1B). These nucleotides are not part of the binding sites of the other factors. Nevertheless, because hydroxyl radical probing by Fe(II)-EDTA tethered to a cysteine in position 700 of EF-G cleaves several residues of the same helix (Wilson & Noller, 1998), the binding site of EF-G in its "fusidic acid stalled state" should be in the proximity of the IF2 binding site. Furthermore, the same loop has been implicated in an interaction with 5S rRNA since RNA–RNA crosslinking experiments have indicated a close proximity between residue U89 of 5S RNA and the conserved residue C2475 (Sergiev et al., 1998).

We also investigated the possibility of an interaction between IF2 and the thiostrepton region around position A1067, which is protected by EF-G. However, IF2 was found to cause no changes in the pattern of chemical probing in this rRNA region (data not shown). Thus, taken together, our data indicate that IF2 interacts with the 50S ribosomal subunit in a fashion similar to but not identical to that of elongation factors EF-G and EF-Tu (Moazed et al., 1988).

IF2 binding to 16S rRNA in 30S subunits and 70S ribosomes

The effect of IF2 on the reactivity of 16S rRNA to chemical probes was then examined in 30S subunits and 70S ribosomes. An IF2-dependent decrease in DMS reactivity was found at A1418 and A1483 in the 16S rRNA of 30S subunits within loose couple 70S ribosomes (Fig. 2, cf. lanes 4 and 5, black arrows) but not in isolated 30S subunits (Fig. 2, cf. lanes 9 and 10). The protected bases are located opposite each other in a bulge in helix 44 and are exposed to DMS modification in isolated 30S subunits (Fig. 2, lanes 9–11) but are protected from modification by the addition of 50S subunits (Moazed & Noller, 1990; Merryman et al., 1999). Under the conditions used by these authors, the two bases in the 30S were reported to retain some reactivity within loose couple 70S ribosomes, and this find-

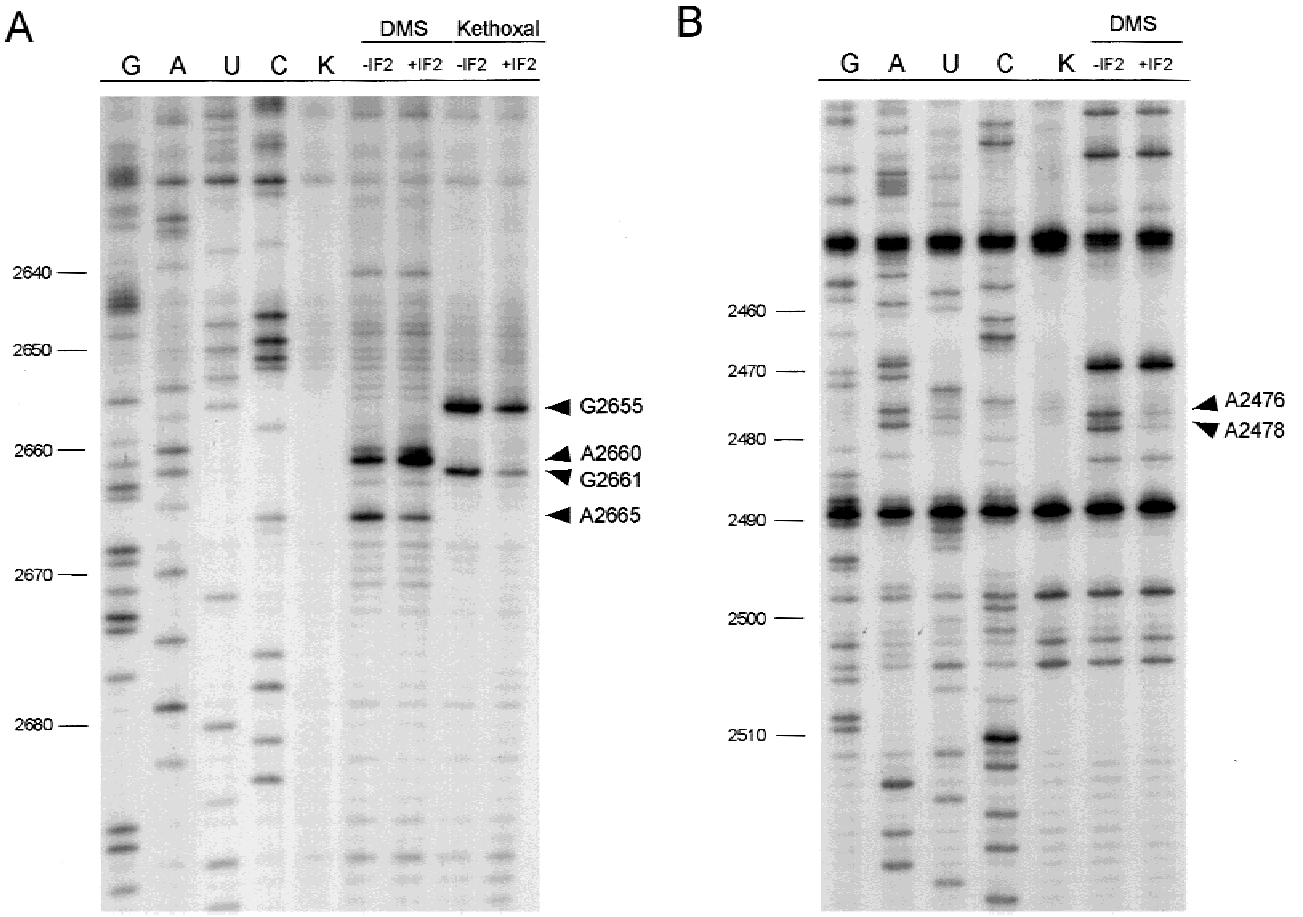

FIGURE 1. Footprinting of *B. stearothermophilus* IF2 on *E. coli* 23S rRNA in loose couple 70S ribosomes (see Materials and Methods). **A:** Protection of bases in the sarcin-ricin domain. **B:** Protection of bases in the loop of helix 89. G, A, U, C: dideoxy sequencing lanes; lane K: unmodified RNA. Modified nucleotides are identified by arrows in right-hand margin.

ing is in agreement with our data (Fig. 2, lane 4). However, because IF2 has no effect on the modification of A1418 and A1483 in isolated 30S subunits (Fig. 2, cf. lanes 9 and 10) and because these bases are not exposed to the modifying agent in "tight coupled" 70S (Fig. 2, lanes 12–14), we conclude that one of the effects of IF2 (like that of the tRNAs) is to strengthen the association between the two ribosomal subunits. This conclusion is supported by the finding that IF1, which can accelerate the rate of subunit exchange (van der Hofstad et al., 1978, and references therein), can partly reverse the IF2 effect (Fig. 2, cf. lanes 5 and 6) and by the finding that the reactivity of A1418 and A1483 decreases upon either A- or P-site binding of tRNA (Moazed & Noller, 1990). A direct demonstration of an increased association of the subunits upon addition of IF2 comes from the results, shown in Fig. 3, of the sucrose density gradient analysis of "loose couple" 70S ribosomes incubated in the absence (panel A) and in the presence (panel B) of IF2. These ribosomes were free of mRNA, tRNA and other translation factors.

Examination of the chemical probing results of Figure 2 (lane 11) reveals that there are several bases (A1408, A1413, A1492, A1493), indicated by the white arrows, whose reactivity is affected by IF1. These results confirm the known topographical localization of IF1 in the A-site (Moazed et al., 1995; Dahlquist & Puglisi, 2000). Furthermore a single base, A1429, located in the lower portion of helix 44 of 16S rRNA, is exclusively accessible to DMS in loose couple 70S ribosomes (Fig. 2, lanes 4–6) regardless of the presence of IF2, and is unmodified in both isolated 30S subunits (Fig. 2, lanes 9–11) and tight couples (Fig. 2, lanes 12–14).

DISCUSSION

Protection from chemical modification of specific sites in 23S rRNA by IF2 is shown here for the first time, and defines more precisely the interaction between IF2 and 23S rRNA. The protection of residues A2476 and A2478 in helix 89 and enhancement of A2660 are unique to IF2, but protection of G2655, G2661, and A2665 in the SRL is similar to that for the elongation factors. EF-Tu protects G2655 and G2661 and weakly protects A2660 and A2665 whereas EF-G protects G2655, A2660, and the 1067 region of domain II.

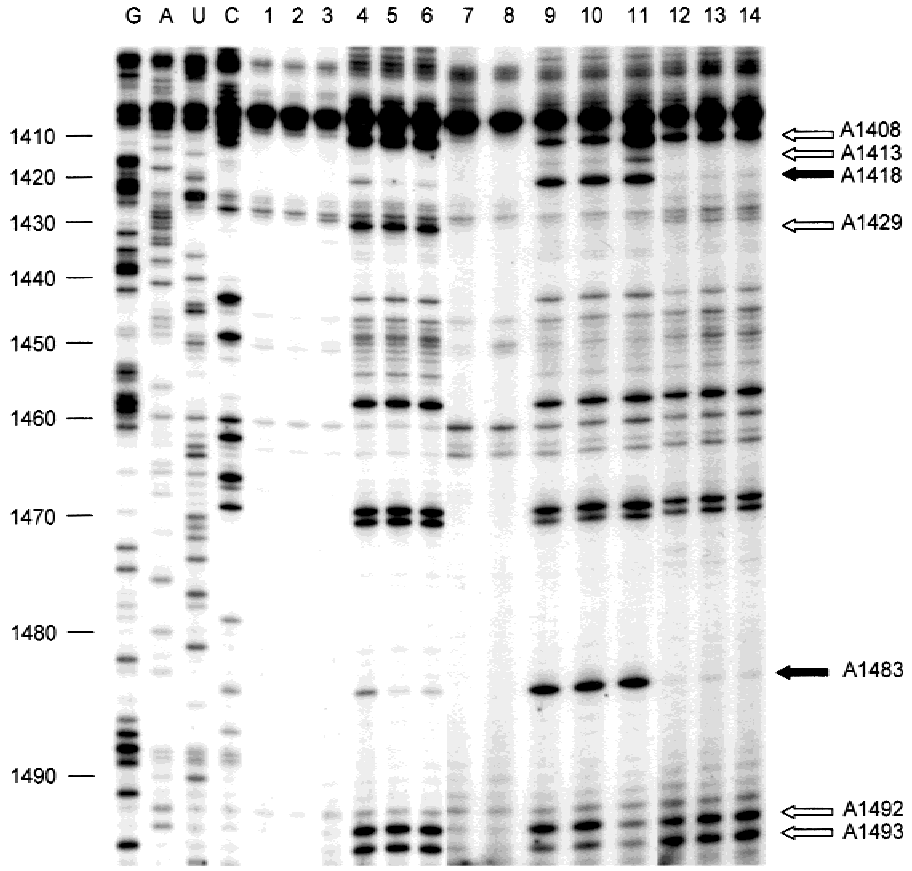

FIGURE 2. Footprinting of *B. stearothermophilus* IF2 and *E. coli* IF1 on *E. coli* 16S rRNA in 30S subunits, loose couple and tight couple 70S ribosomes. G, A, U, C are dideoxy sequencing lanes. Lanes 1, 2, and 3: unmodified 16S rRNA from loose couple 70S alone (1), in the presence of IF2 (2) and in the presence of IF1 and IF2 (3). Lanes 4, 5, 6: DMS-modified RNA from loose couples 70S alone (4), in the presence of IF2 (5) and in the presence of IF1 and IF2 (6). Lanes 7, 8: unmodified RNA from 30S (7) and from tight couple 70S (8). Lanes 9, 10, 11: DMS-modified RNA from 30S alone (9), in the presence of IF2 (10), in the presence of IF1 and IF2 (11). Lanes 12, 13, 14: DMS-modified RNA from tight couple 70S alone (12), in the presence of IF2 (13), and in the presence of IF1 and IF2 (14).

The overlap between the ribosomal binding sites of elongation factors EF-G and EF-Tu was originally proposed on the basis of the inability of the two factors to bind simultaneously to the ribosome (Richman & Bodley, 1972) and further confirmed by their topographical localization (Moazed et al., 1988). A similar overlap between the binding sites of IF2, EF-G, and EF-Tu has never been convincingly demonstrated. Indeed, the assumption that such overlap exists was based mainly on evidence derived from protein–protein crosslinking experiments (Heimark et al., 1976; Boileau et al., 1983).

Under the same conditions, which allow P-site protection of 23S rRNA by tRNA, IF2 did not affect the reactivity of any nucleotides in 16S rRNA. In fact, the effects seen on A1418 and A1483 are likely indirect and, as mentioned above, indicate that IF2 favors subunit association, a conclusion supported by the sucrose density gradient experiment shown in Figure 3. The lack of protection of 16S rRNA in 70S ribosomes

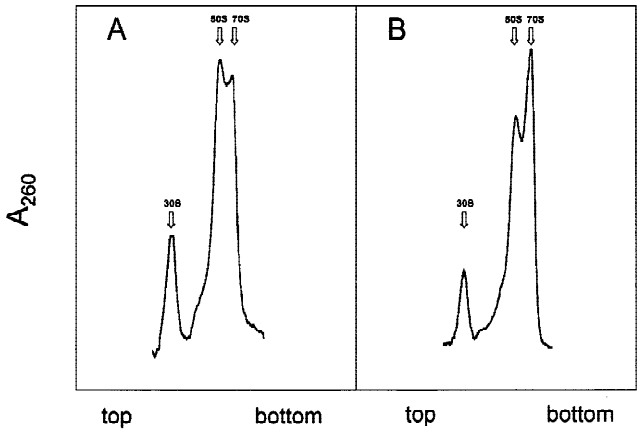

FIGURE 3. Effect of *B. stearothermophilus* IF2 on subunit association. Heat activated loose couple 70S ribosomes incubated in the absence (**A**) and in the presence (**B**) of *B. stearothermophilus* IF2 were separated by sucrose density gradient centrifugation as described in Materials and Methods. The subunits and ribosomes are indicated by 30S, 50S, and 70S.

can be explained by the fact that, in the presence of 50S subunits, IF2 may lose its contact(s) with the 30S subunits or the IF2-30S interaction may be weakened. However IF2 does not protect 16S rRNA in isolated 30S subunits, and this finding raises the question of how IF2 interacts with the 30S subunits, namely via contacts with ribosomal proteins or through several weak interactions with 16S rRNA. The latter possibility is suggested by the results of Wakao et al. (1990), who found changes of chemical reactivity of 16S rRNA caused by IF2 to be scattered throughout the entire molecule and concluded that IF2 does not protect specific regions but induces allosteric transition in the rRNA.

The three-dimensional structure of the *Haloarcula marismortui* 50S subunit was solved recently at a resolution of 2.4 Å (Ban et al., 2000). This allows us to localize in space the IF2 binding site. Figure 4 shows the crystal structure of *H. marismortui* 23S rRNA from Ban et al. (2000), as seen from the subunit interface side. In this structure, the *H. marismortui* residues corresponding to those of *E. coli* involved in IF2 binding have been highlighted: Residues protected from DMS modification, A2511 and A2513 (corresponding to A2476 and A2478 in *E. coli*) in helix 89 and A2702 (A2665 in *E. coli*) in the α-sarcin domain, are shown in red; residue A2697 (A2660 in *E. coli*), whose reactivity is enhanced, is shown in yellow, and residues protected from kethoxal modification, G2692 and G2698 (G2655 and G2661 in *E. coli*), are shown in blue. These 23S rRNA bases are located just below L11, on the right edge of the subunit interface side of the 50S particle. The positions affected by IF2 are relatively close to each other

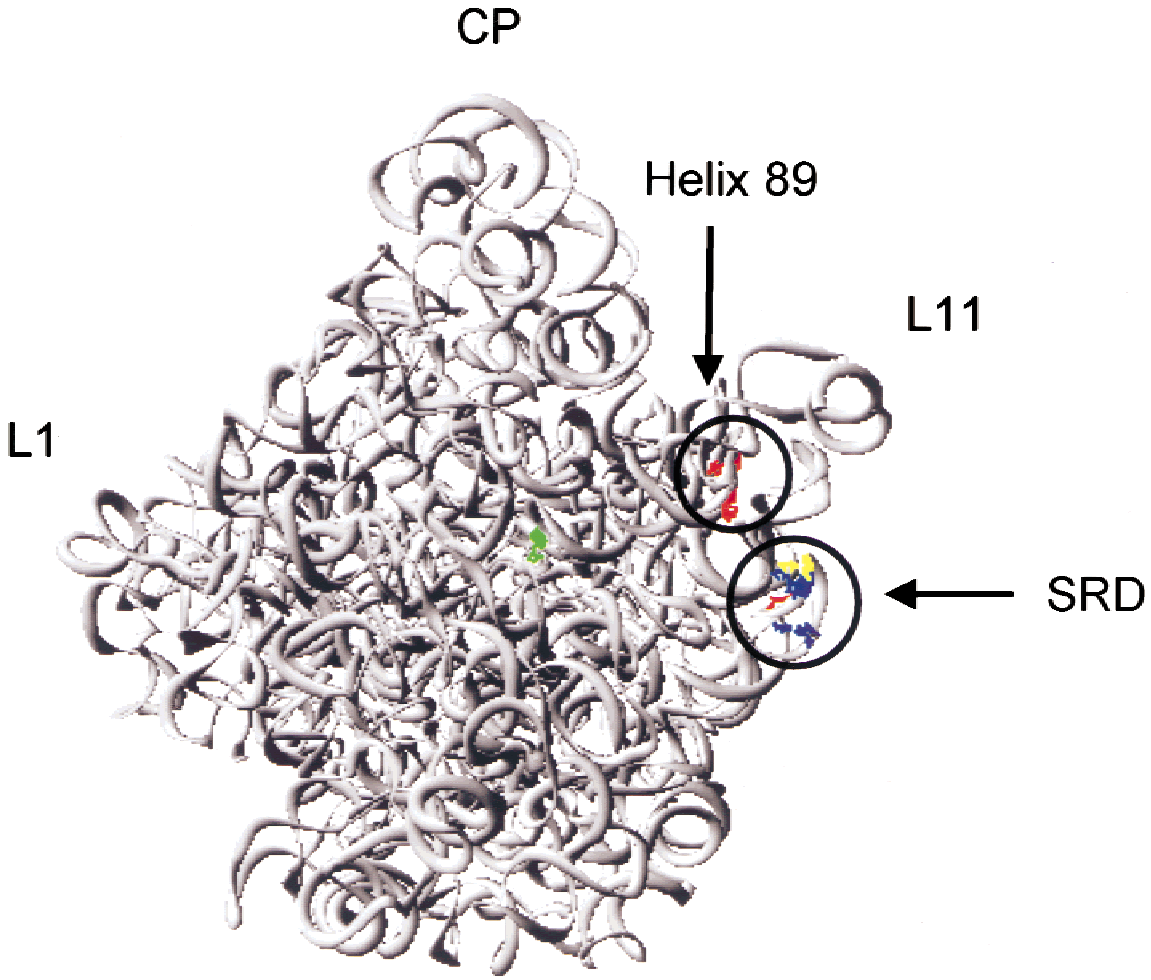

FIGURE 4. Crystal structure of *H. marismortui* 23S rRNA from Ban et al. (2000) as seen from the subunit interface side. Residues protected by IF2 from DMS modification, A2511 and A2513 (corresponding to A2476 and A2478 in *E. coli*) in helix 89 and A2702 (A2665 in *E. coli*) in the SRD, and residues protected from kethoxal modification, G2692 and G2698 (G2655 and G2661 in *E. coli*), are shown in red and blue, respectively; A2697 (A2660 in *E. coli*) at the tip of the SRD, whose reactivity towards DMS is enhanced by IF2, is highlighted in yellow. Residue A2486 (A2451 in *E. coli*) is shown in green. The structural coordinates of the 50S subunit (Ban et al., 2000) have been taken from the Protein Data Bank and the colors modified using the Swiss-PDB Viewer 3.7 program. Positions of L1 and L11 ribosomal proteins are marked; CP: central protuberance; SRD: sarcin-ricin domain.

but fairly distant from the peptidyl transferase center. In fact, the latter, which has been identified by Nissen et al. (2000) as residue A2486 (A2451 in *E. coli*), shown in green in Figure 4, is located 53.5 Å from A2476 and 74.7 Å from position A2660 (R. Brimacombe, pers. comm.). On the other hand, the peptidyl transferase center is the site where the acceptor end of initiator tRNA (fMet-ACC) must bind to allow the formation of the initiation dipeptide. It has been shown recently that IF2 C-2, the C-terminal domain of IF2, contains all molecular determinants for the binding of initiator tRNA (Meunier et al., 2000; Spurio et al., 2000). Thus the C-terminal domain of IF2, which does not bind autonomously to any ribosomal subunit (Gualerzi et al., 1991), must reach the peptidyl transferase center, which is located in a central point of the particle, whereas the contacts of IF2 with the 23S rRNA must be established by parts of the G-domain that retain ribosomal binding capacity. Thus we conclude IF2 must be a rather elongated molecule spanning at least from the center to the edge of the 50S subunit. This prediction is supported by the three-dimensional structure of the archaeal homolog of bacterial IF2 recently elucidated (Roll-Mecak et al., 2000).

Recent fluorescence stopped-flow experiments have demonstrated that an EF-Tu-GTP-Phe-tRNA ternary complex binds to the ribosomal A-site of 70S initiation complexes with the same kinetics in the presence or absence of IF2-dependent GTP hydrolysis (Tomsic et al., 2000). The results shown in the present article indicate that, upon interaction with the 70S ribosomes, IF2 establishes contacts with the 23S rRNA that are, at least in part, the same as those made by elongation factors EF-G and EF-Tu, indicating that the binding sites of these factors are indeed overlapping. In turn, this result allows us to conclude that the ejection of IF2 from the ribosome or its recycling off the ribosomes does not require the GTPase activity of the initiation factor as recently reported (Luchin et al., 1999). In fact, overlapping IF2/EF-Tu sites should have resulted in an inhibition of EF-Tu binding in the absence of IF2-dependent GTP hydrolysis, a result in contrast to that actually obtained (Tomsic et al., 2000). The function of IF2 GTPase, therefore remains obscure. However recent data have shown that the eukaryotic (and presumably archaeal) homolog of bacterial IF2 (initiation factor eIF5B), promotes subunit association and is endowed with a ribosome-dependent GTPase activity that is necessary for this function (Lee et al., 1999; Pestova et al., 2000). Our chemical probing and sucrose density gradient results, along with previous light-scattering studies (Grunberg-Manago et al., 1975) indicating that IF2 favors the association of bacterial subunits, are particularly interesting given the universal structural conservation of this factor and its functional homology with other translation factors.

MATERIALS AND METHODS

General preparations

30S and 50S ribosomal subunits, 70S ribosomes (called loose couple 70S ribosomes), and fMet-tRNA were prepared and purified according to standard procedures (Ohsawa & Gualerzi, 1983) and are free of tRNA, mRNA, and translational factors. Tight couple 70S ribosomes were purified in sucrose density gradients (10%–30%) in 10 mM Tris-HCl, pH 7.7, 6 mM Mg acetate, 60 mM NH_4Cl, 1 mM DTT (described below). *Bacillus stearothermophilus* IF2 was prepared from an overproducing strain and purified as described (Brombach et al., 1986).

Preparation of ribosome-IF2 complexes

For the preparation of the complexes, 50 pmol of 70S ribosomes were preactivated by incubation for 20 min at 42 °C in 80 mM K-cacodylate, pH 7.2, 10 mM $MgCl_2$, 100 mM NH_4Cl, 1.5 mM DTT. The 70S-IF2 complexes were then prepared incubating (10 min at 37 °C), in the same buffer, the 70S ribosomes in the presence of 200 pmol of *B. stearothermophilus* IF2. "70S initiation complexes" were prepared under the same conditions with the additional presence of 100 pmol IF1, 100 pmol fMet-tRNA, 5 μg random poly (AUG) and 1 mM GTP.

Chemical probing

The modification reactions were carried out according to Moazed et al. (1986) using dimethylsulphate (DMS, Kodak), kethoxal (Research Organics) and CMCT (Sigma). After modification, ribosomal RNA was extracted (Moazed et al., 1986) and the modified residues identified by primer extension analysis.

Primer extension

Two picomoles of the selected primer, 5′-^{32}P labeled with T4 kinase, were incubated with 2 μg of the modified rRNA for 3 min at 70 °C in 10 μL of 50 mM Tris-HCl, pH 8.3, 40 mM KCl and then slowly cooled to 40 °C. After addition of 2 U of AMV Reverse Transcriptase (Life Science; diluted in 50 mM Tris-HCl, pH 8.3, 40 mM KCl, 6 mM Mg_2Cl) 2 μL of each annealing reaction were mixed with 2 μL of a 2× extension mixture (100 mM Tris-HCl, pH 8.3, 80 mM KCl, 12 mM Mg_2Cl, 4 mM of each deoxynucleotide triphosphate) and incubated for 30 min at 45 °C. The extension reaction was stopped by addition of 2 μL formamide/dye buffer and 2.5 μL were loaded on a 10% sequencing gel.

Subunit association analysis

Heat-activated (20 min at 42 °C) *E. coli* 70S ribosomes (22 pmol) were incubated for 10 min at 37 °C in 100 μL of 10 mM Tris-HCl, pH 7.7, 100 mM NH_4Cl, 3 mM Mg acetate, 1 mM DTT, in the presence and absence of *B. stearothermophilus* IF2 (135 pmol). The samples were then loaded onto sucrose gradients (10%–30% in the same buffer) and centrifuged in a

SW40 Beckman rotor at 20,000 rpm for 17 h at 3 °C. Gradients were collected using an ISCO gradient fractionator.

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