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Identification of the ribosome binding sites of translation initiation factor IF3 by multidimensional heteronuclear NMR spectroscopy

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ABSTRACT

Titrations of *Escherichia coli* translation initiation factor IF3, isotopically labeled with ^{15}N, with 30S ribosomal subunits were followed by NMR by recording two-dimensional ($^{15}N,^{1}H$)-HSQC spectra. In the titrations, intensity changes are observed for cross peaks belonging to amides of individual amino acids. At low concentrations of ribosomal subunits, only resonances belonging to amino acids of the C-domain of IF3 are affected, whereas all those attributed to the N-domain are still visible. Upon addition of a larger amount of 30S subunits cross peaks belonging to residues of the N-terminal domain of the protein are also selectively affected.

Our results demonstrate that the two domains of IF3 are functionally independent, each interacting with a different affinity with the ribosomal subunits, thus allowing the identification of the individual residues of the two domains involved in this interaction. Overall, the C-domain interacts with the 30S subunits primarily through some of its loops and α-helices and the residues involved in ribosome binding are distributed rather symmetrically over a fairly large surface of the domain, while the N-domain interacts mainly via a small number of residues distributed asymmetrically in this domain.

The spatial organization of the active sites of IF3, emerging through the comparison of the present data with the previous chemical modification and mutagenesis data, is discussed in light of the ribosomal localization of IF3 and of the mechanism of action of this factor.

Keywords: 30S ribosomes; NMR titrations; protein domains; protein synthesis; RNA–protein interaction

INTRODUCTION

Translational initiation factor IF3 is one of the three proteins required for initiation of protein synthesis in bacteria (for reviews, see Gualerzi & Pon, 1990; McCarthy & Gualerzi, 1990). In *Escherichia coli*, IF3 consists of 180 amino acids encoded by *infC*, a gene mapped at 37.5 min (Sacerdot et al., 1982) and proven to be essential for cell survival (Olsson et al., 1996).

Acting as a subunit anti-association factor, IF3 supplies the pool of free 30S subunits required for translation initiation (Hershey, 1987, and references therein). Furthermore, IF3 accelerates the formation of 30S in-

tiation complexes and ensures the fidelity of translation initiation promoting the dissociation of noncanonical complexes (i.e., complexes containing aminoacyl-tRNAs other than initiator fMet-tRNA and/or a triplet other than the initiation triplets AUG, GUG, and UUG) (Gualerzi et al. 1971; Pon & Gualerzi, 1974; Hartz et al., 1989; Haggerty & Lovett, 1993; La Teana et al., 1993; Sussman et al., 1996). This function can be accounted for by the influence of IF3 on both on and off rates of codon–anticodon interaction in the P-site (Gualerzi et al., 1977; Wintermeyer & Gualerzi, 1983; Hartz et al., 1989) and possibly by an IF3-induced adjustment of the mRNA that is shifted from the "stand-by site" to the "P-decoding site" on the 30S ribosomal subunit (La Teana et al., 1995).

The interaction of IF3 with the ribosome was one of the first translational functions attributed to the 16S

rRNA moiety of the ribosome (for a review, see Santer & Dahlberg, 1996). Based on the topographical localization of IF3 on the 30S ribosomal subunit as determined by immunoelectron-microscopy, protein–protein and protein–RNA crosslinking, and the structural and functional properties of IF3 available at that time, Pon et al. (1982b) suggested that IF3 bridges the cleft of the small ribosomal subunit by binding with two separate sites to both head and platform, and that it fulfills its functional role by affecting the conformational dynamics of the ribosome.

The two-domain nature of IF3 has recently been confirmed by structural investigations. The 3D structures of the separate domains of *Bacillus stearothermophilus* IF3 have been elucidated by X-ray crystallography (Biou et al., 1995) and those of *E. coli* IF3 by NMR spectroscopy (Fortier et al., 1994; Garcia et al., 1995a, 1995b). In intact IF3 these two domains of the molecule are separated by a fairly long and flexible linker (Moreau et al., 1997).

In this article we have made use of multidimensional heteronuclear NMR spectroscopy to study the interaction of intact IF3 with its complete natural target, the 30S ribosomal subunit. Our results provide evidence that both domains of IF3 interact, albeit with different affinity, with the 30S ribosomal subunit and allow us to identify the amino acid residues of IF3 involved in this interaction.

RESULTS

The two domains of IF3 are functionally separate

Prior to the "30S-titration" experiments three-dimensional (3D) TOCSY-(^{15}N,^{1}H)-HSQC and 3D NOESY-(^{15}N,^{1}H)-HSQC spectra of ^{15}N-labeled purified *E. coli* translation initiation factor IF3 were recorded at 750 MHz (data not shown) to identify, using the reported assignments of the separate N- and C-domains (Garcia et al., 1995a, 1995b), the individual resonances of the intact 180 residues containing protein. The chemical shifts of the amide backbone atoms of intact IF3 (supplementary material, available from the authors upon request) are essentially the same as those of the individual domains, indicating that in free IF3 few (if any) interactions exist between them. To study the IF3-30S interaction, two-dimensional (2D) (^{15}N,^{1}H)-HSQC spectra of IF3 were recorded before and after the progressive addition of small aliquots of *E. coli* 30S ribosomal subunits (M.W. = 900 kDa) to the NMR tube containing a constant amount of protein. These titration experiments were repeated twice yielding consistent results. The spectrum of the free protein (Fig. 1A) displays many resolved signals, with most of the amide protons being visible, in spite of the fairly high pH (6.5) used to avoid precipitation of the ribosomes. Cross peaks in the spec-
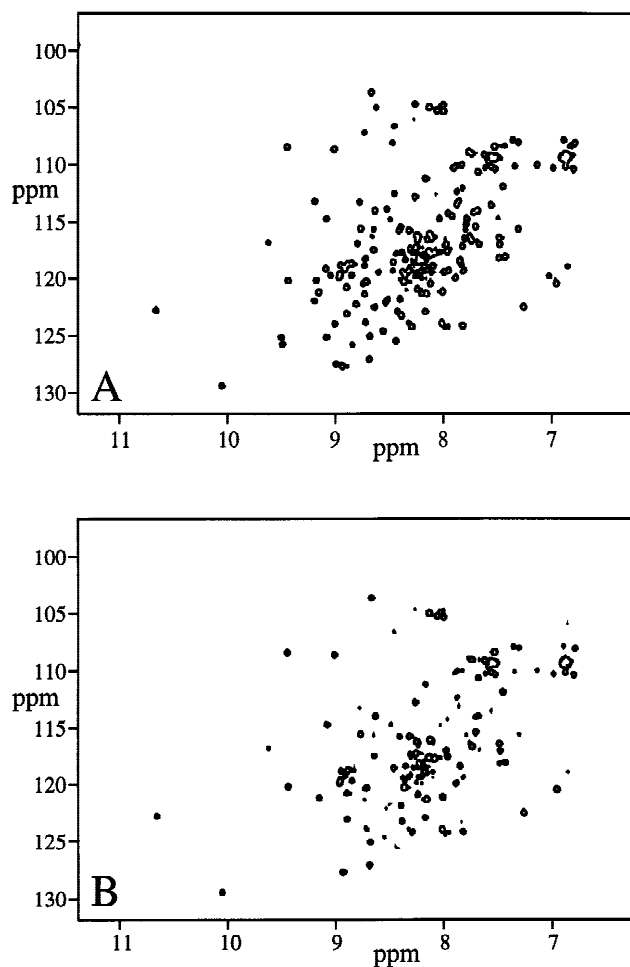

FIGURE 1. 2D (^{15}N,^{1}H)-HSQC spectra of free and ribosome-bound *E. coli* IF3. The spectra of a 2-mM solution of ^{15}N-labeled IF3 were recorded in the absence (**A**) and in presence (**B**) of *E. coli* 30S ribosomal subunits at a stoichiometric ratio 30S/IF3 of 0.02%.

tra represent either the backbone amides of the individual amino acids or the side-chain amides of asparagines or glutamines. Thus, the quality of the spectra is such that a large portion of the protein can be monitored. Spectral changes observed after the addition of 30S ribosomal subunits (one of these spectra is presented in Fig. 1B as an example) can provide direct and meaningful information concerning the regions of the molecule involved in the interaction with the ribosomes.

In fact, the (^{15}N,^{1}H)-HSQC spectra recorded in the presence of increasing amounts of ribosomal subunits showed that the intensities of some cross peaks of IF3 are decreased due to line broadening (cf. Fig. 1A and 1B). Upon complex formation, residues, which are involved in the interaction with the ribosomes, may give rise to considerable shifts in their corresponding resonances versus those of free IF3. The addition of low amounts of ribosomal subunits (e.g., in a ratio of 1:1,000) in the presence of a suitable salt concentration establishes a medium-to-fast exchange equilibrium between

free and bound IF3, which mainly leads to selective line broadening without significant effect on the resonance position. The analysis of the relative change in cross-peak intensity, as shown in the histograms presented in Figures 2 and 3, allowed us to identify those amino acids of the C-domain (Fig. 3A) and of the N-domain (Fig. 3B) that are affected most by the addition of the ribosomal subunits, as well as those that are affected little or not at all. Though the specific changes for individual amino acids could be observed throughout a complete titration series, the largest discrimination between specific and more global changes was observed at low 30S/IF3 ratios. Continued addition of 30S subunits led to the complete disappearance of all cross peaks.

The titration experiments revealed that the IF3 molecule behaves as if it consists of two functionally independent domains, each interacting with the ribosomal subunits with a different affinity (see Discussion). In fact, the amino acid resonances belonging to the C-domain of the protein were affected sooner and to a larger extent then those belonging to the N-domain and it is possible to select a 30S/IF3 ratio at which only the resonances due to the amino acids belonging to the C-domain are specifically broadened while the resonances belonging to the N-domain remain fairly unaffected (Figs. 1B and 2). The complete immobilization of the residues belonging to the N-terminal part of the molecule was observed only when the 30S/IF3 ratio was increased by further additions of ribosomal subunits.
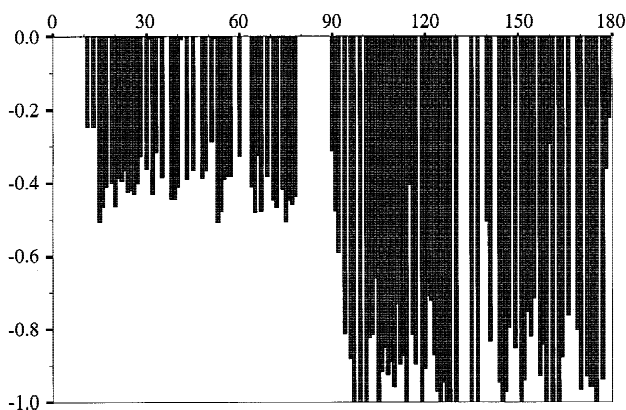

FIGURE 2. Effect of addition of 30S ribosomal subunits on the backbone amide resonances of the N-terminal and C-terminal domain of IF3. The figure shows differential intensity changes for the C-terminal and N-terminal domains. The position of each amino acid in the primary sequence of IF3 is indicated in the abscissa; the ordinate presents the relative intensity change, $(V - V_0)/V_0$, of each assigned cross peak caused by the addition of *E. coli* 30S ribosomal subunits at a stoichiometric ratio 30S/IF3 of 0.02%. The blank slots correspond to residues for which the intensity could not be determined because of the absence of their signal (i.e., for Pro residues and in case of fast exchange) or because of severe overlap. Further experimental details are given in Materials and Methods.
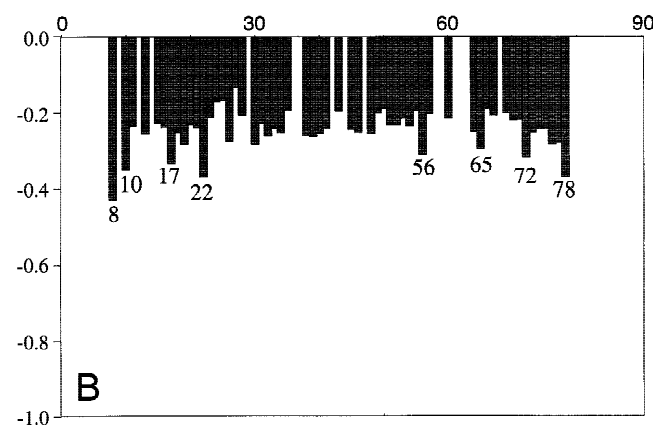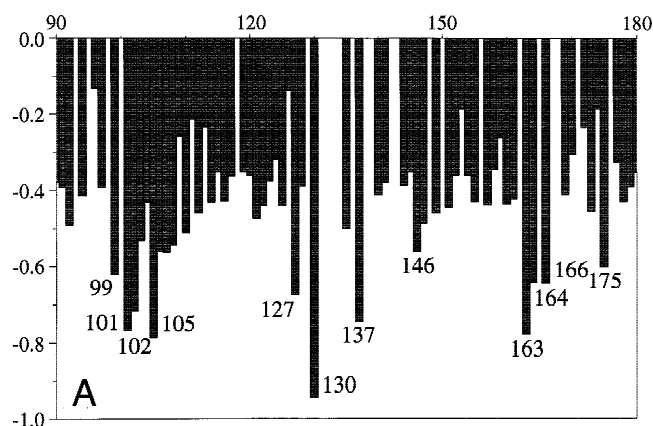

FIGURE 3. Intensity changes of individual backbone amide resonances of IF3 upon addition of 30S ribosomal subunits. **A** and **B** show the relative intensity change caused by successive additions of 30S ribosomal subunits for residues assigned to the C-domain (residues 90–180) and N-domain (residues 8–78) of IF3, respectively. The stoichiometric ratio 30S/IF3 was 0.002% for the data presented in **A** and 0.01% for **B**. The blank slots correspond to residues for which the intensity could not be determined because of the absence of their signal (i.e., for Pro residues and in case of fast exchange) or because of severe overlap. Further details are given in Materials and Methods.

Identification of the amino acids of IF3 implicated in the interaction with 30S subunits

The amino acids affected by the addition of the ribosomal subunits, consistently observed at increasing 30S/IF3 ratios, are compiled in a scheme that correlates their localization with the known elements of secondary structure (Fig. 4) and are highlighted in the 3D structures of the C-domain (Fig. 5A) and N-domain (Fig. 5C).

Structure determination of the C-domain of IF3 by NMR (Garcia et al., 1995b) has shown that this domain is composed of four β-strands forming a β-sheet which is located behind two α-helices (H3 and H4). Figures 4 and 5A, which summarize the results of the complete titration of IF3 with 30S, show that most of the C-domain residues involved in the interaction with the 30S particles are located in helix H3 (Asp106, Gln108, Lys110,
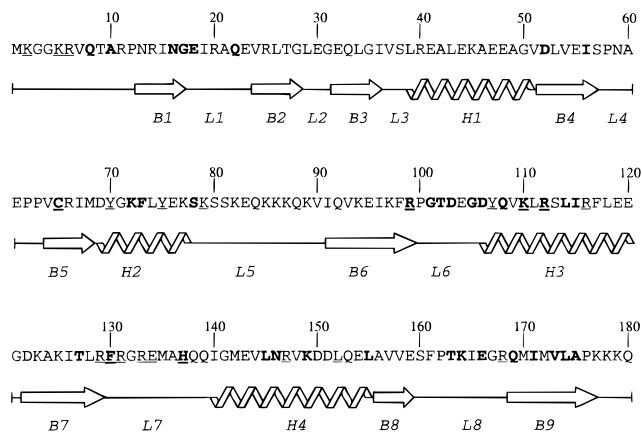
FIGURE 4. Amino acid residues of IF3 implicated in the interaction with the 30S ribosomal subunit. The amino acid residues most affected in the experiments by the addition of 30S ribosomes to IF3 and therefore involved in 30S interaction are indicated (in bold) within the primary sequence of IF3. The identification of the most affected residues was based on a decrease in intensity that was significantly and consistently larger than that of the bulk of the residues. Underlined residues indicate amino acids that were identified by mutagenesis and/or chemical modification. Known elements of secondary structure are indicated as reported by Garcia et al. (1995a, 1995b). See text for further details.

Arg112, Leu114, and Ile115) and in part of helix H4 (Leu145, Asn146, Lys149, and Leu155) thus forming one side of the domain. Other residues involved in interaction are located in the loops L6 (Arg99, Gly101, Thr102, Asp103, and Gly105), L7 (Phe130, His137), and L8 (Thr163, Lys164, and Glu166) and the β-strands B7 (Thr127) and B9 (Gln169, Ile171, Val173, Leu174, and Ala175). In the 3D structure most of these residues are located on one side of the C-domain (i.e., that facing the observer) and define a fairly extended ribosomal binding site (Fig. 5A).

The structural data concerning the N-domain (Garcia et al., 1995a) have indicated that this domain is composed of five β-strands forming a β-sheet in front of which there is an α-helix connecting the third and the fourth β-strand; the latter β-strand is followed by a second α-helix that precedes a 13-amino-acid-long hydrophilic, positively charged, and highly mobile linker connecting this domain with the C-domain (Moreau et al., 1997; see below). The present NMR titration data show that the residues of the N-domain involved in the binding (Figs. 4 and 5C) are mainly localized in the N-terminal tail (Glu8, Ala10), in β-strands B1 (Asn16 and Gly17), B4 (Asp52, Ile56), and B5 (Cys65) in the loop (L1) connecting strands B1 and B2 (Glu18 and Gln22) in the C-terminal helix H2 (Lys72, Phe73) and in the start of the linker (Ser78). These elements are distributed asymmetrically in the 3D structure of the N-domain and are found relatively close to each other, defining a rather small (compared to that of the C-domain) interacting surface. The structure of the linker connecting the N-terminal and C-terminal domains has

not been unambiguously characterized (Biou et al., 1995; Garcia et al., 1995a; Moreau et al., 1997). In fact, the linker consists of seven residues in the crystal structure of *B. stearothermophilus* IF3 and of 13 amino acids in the solution structure of *E. coli* IF3, as determined by NMR spectroscopy. The reason for this discrepancy can be traced back to the different lengths attributed to the last α-helix (H2) of the N-domain in the two models. It should be noted, however, that most of the NMR spectroscopy studies on *E. coli* IF3 have thus far been carried out on the two separated domains (in these studies the linker was associated with the N-terminal domain and was absent in the C-domain) and that the resonances due to the linker could not be assigned in the NMR spectrum, because of the very flexible nature of this portion of the molecule. This flexibility was later confirmed by the relaxation times of the ^{15}N-labeled (unassigned) lysines belonging to the linker. In the 3D spectra recorded on the full *E. coli* protein, we were able to identify Ser78 and Lys89 but failed to assign the other 11 residues separating these two amino acids. Thus, our spectral data confirm the premise that the linker corresponds to a region endowed with high flexibility, whereas the titration data indicate that within the linker region, at least Ser78 is strongly affected by the interaction with the 30S particle.

DISCUSSION

We have described here experiments aimed at the identification of the specific amino acid residues of IF3 involved in the interaction of this protein with the 30S ribosomal subunits. This work, in which we have used the resolution of 2D heteronuclear NMR spectroscopy, was inspired by the promising results obtained when changes in the IF1 (Paci et al., 1983) and IF3 (Paci et al., 1985) one-dimensional ^{1}H-NMR spectra were monitored following the addition of small amounts of deuterated 30S ribosomal subunits and by the recent results in the identification of the active site of IF1 by 2D heteronuclear NMR spectroscopy (Sette et al., 1997). The identification of the active residues of IF3 was also made possible by the recent elucidation of the 3D structures of this protein in *E. coli* and *B. stearothermophilus* (Biou et al., 1995; Garcia et al., 1995a, 1995b; Kycia et al., 1995).

Attempts to identify the amino acid residues of IF3 involved in the interaction with the 30S subunit, as well as the site(s) of the ribosomal subunit active in binding IF3 (see below), have been carried out in the past, mainly by selective chemical modifications, site-directed mutagenesis, crosslinking reactions, and chemical or enzymatic probing. Several lines of evidence had indicated that IF3 contains two active sites interacting with two separate regions of the 30S ribosomal subunit and it was suggested that a stable interaction of one site and a fluctuating interaction of the other could affect the conformational dynamics of the 30S ribosomal sub-
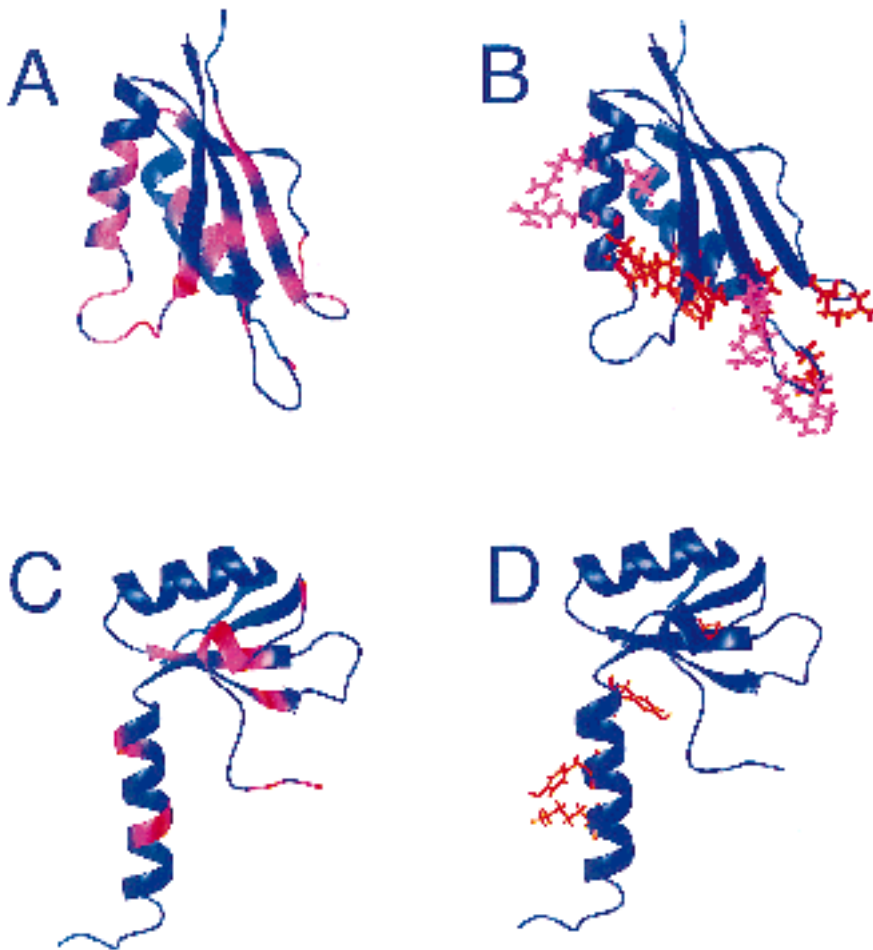
FIGURE 5. Ribbon diagrams of the C-domain (**A**, **B**) and N-domain (**C**, **D**) of IF3, highlighting amino acid residues involved in interaction with the 30S ribosomal subunit. Interacting residues, as defined by intensity changes in the NMR spectra, are shown in **A** and **C**, and are colored red and purple, where red indicates the strongest effects. Interacting sidechains, as identified by mutagenesis and chemical modification, are shown in **B** and **D**, and are colored red and purple, where red indicates the strongest effects. The coordinates of the structures were obtained from the Protein Data Bank. The C-domain is the NMR structure of the *E. coli* protein (PDB entry: 1IFE; Garcia et al., 1995a) and the N-domain is an energy-minimized structure based upon the backbone coordinates of the X-ray structure of *B. Stearothermophilus* protein (PDB entry: 1TIG; Biou et al., 1995), as described in Materials and Methods. Drawings were made with the program MOLMOL (Koradi et al., 1996).

unit, thus accounting for the biological effect of IF3 on the association-dissociation of the codon-anticodon interaction in the ribosomal P-site (Pon et al., 1982b).

The existence of two separate domains, approximately 46 Å apart (Kycia et al., 1995), has been fully established by the recent structural studies. The evidence presented here that the resonances belonging to the amino acids of the C-domain are immobilized by the 30S ribosomal subunit before those belonging to the N-domain (Fig. 2) clearly indicates that the two domains are not only structurally but also functionally separated in their interaction with the ribosomes. In fact, the behavior of IF3 in these experiments is compatible with the idea that in the NMR tube there are three species of IF3: free (F-IF3), partly (through the C-domain) bound (PB-IF3), and fully (through both C-domain and N-domain) bound (FB-IF3).

Upon addition of very low amounts of 30S subunits, the establishment of a medium-fast exchange equilibrium between F-IF3 and PB-IF3 causes the selective broadening of the cross peaks belonging to the C-domain. Through a first-order isomerization of the initial IF3-30S complex, a portion of PB-IF3 becomes FB-IF3; as the concentration of PB-IF3 increases the

level of FB-IF3 also increases and the interactions with the N-domain start being detectable through the broadening of the corresponding cross peaks.

The identification by NMR of a number of amino acid residues belonging to both C- and N-domain that are affected by the addition of 30S ribosomal subunits (Fig. 4, residues written in bold characters) represents an additional contribution to the elucidation of the mechanism of the IF3-30S interaction. In many cases, these amino acids are the same as or adjacent to those previously identified by both biochemical and genetic approaches as being involved in the IF3-30S interaction (Fig. 4, residues underlined). It seems therefore both appropriate and useful to discuss all these data together with the present NMR results. The relevant residues are summarized and highlighted in the 3D structures of the C-domain (Fig. 5A,B) and N-domain (Fig. 5C,D).

Nature of the ribosome binding site of the C-domain

The finding that the C-domain is affected by the addition of the ribosomal subunit more readily and more

intensely than the N-domain is in full agreement with the localization of the primary RNA binding site of IF3 within this region of the molecule. Thus, based on the present NMR data and on previous results, three main regions of the C-domain can be singled out as being important for the interaction with the 30S ribosomal subunit. The first of these regions, spanning from Arg99 through Arg116, is constituted by the distal tip of strand B6, by the loop (L6) connecting strand B6 and helix H3 and by the proximal half of H3. Nearly all residues in this region are heavily involved in the interaction of IF3 with the 30S subunit. In fact, this region of the molecule contains Arg99, Gly101, Thr102, Asp103, Gly105, Asp106, Gln108, Lys110, Arg112, Leu114, and Ile115, which were shown to be involved by NMR spectroscopy (Figs. 4 and 5A). Furthermore, other approaches have identified some of the same residues (Arg99, Lys110, and Arg112), as well as additional ones (Tyr107 and Arg116; Fig. 5B). In fact, iodination of Tyr107 (Bruhns & Gualerzi, 1980), modification of Lys110 with pyridoxal phosphate (Ohsawa & Gualerzi, 1981), and amino acid substitutions at the same positions by site-directed mutagenesis (De Bellis et al., 1992) were found to reduce the association constant of IF3 for the 30S ribosomal subunit severely. Furthermore, site-directed mutagenesis of Arg99 and Arg116 demonstrated that these residues are also very important for this interaction (Petrelli et al., 1998; R. Spurio & C.O. Gualerzi, unpublished results). A functional role for Arg99 is also indicated by the identification of two spontaneous mutants with defective activity, *srjA3* and *srjA4*, in which this residue is replaced by His or Leu, respectively (Haggerty & Lovett, 1993). Arg 112, on the other hand, has not yet been mutated or modified in *E. coli* IF3, but an Arg103 mutant of *B. stearothermophilus* IF3 (which corresponds to Arg112 of *E. coli*) displays reduced binding and reduced activity (Petrelli et al., 1998; R. Spurio & C.O. Gualerzi, unpublished results).

This region of the molecule probably represents the primary RNA binding site of IF3. In fact, the NMR spectra show that the residues belonging to this part of the molecule are among the first and most affected by the addition of 30S subunits and mutation and/or chemical modification of at least some of these residues was found to affect directly the binding of IF3 to the 30S subunit whereas both 30S subunit and 16S rRNA were found to protect this region of the molecule from chemical modifications (Bruhns & Gualerzi, 1980; Ohsawa & Gualerzi, 1981). The validity of this premise is further strengthened by the structural homologies with other nucleic acid binding proteins (see below).

The second region of the C-domain that is clearly implicated in the functional interaction with the 30S ribosomal subunit is that comprised between Thr127 and His137 and between Leu145 and Leu155 (Figs. 4 and 5A,B). Unfortunately, only a few of the residues between position 138 and 144 are unambiguously assigned in our NMR spectra and therefore we cannot come to firm conclusions concerning the involvement of these amino acids. As shown by the NMR results summarized in Figures 4 and 5A, the region involved in the ribosomal binding consists of the distal portion of B7, most of the loop L7 and the distal half of helix H4 with residues Thr127, Phe130, His137, Leu145, Gln146, Lys149, and Leu155; furthermore, mutagenesis of Arg129, Phe130, Arg133, and Arg147 resulted in IF3 molecules with impaired activity (Spurio et al., in prep.), while His137 was found to be essential for the IF3-30S interaction following rose bengal-sensitized photo-oxidation (Lammi et al., 1982). Finally, an in vitro-generated mutation in Cys144 in *B. stearothermophilus* IF3 (corresponding to Leu152 of *E. coli*) reduced the activity of the factor, probably by destabilizing helix H4 (Petrelli et al., 1998; R. Spurio & C.O. Gualerzi, unpublished results) and spontaneous mutations, in which Arg131 was replaced by Pro (Haggerty & Lovett, 1993) and Glu134 by Lys (Sacerdot et al., 1996), resulted in partly defective IF3 molecules. Though important for binding to the ribosome, loop L7 (or at least part of it) is probably not included in the main 16S rRNA binding site of the IF3 C-domain because the Glu134 mutant is only partially defective (Sacerdot et al., 1996) and His137, unlike the residues belonging to the "primary RNA binding site" (e.g., Arg99, Tyr107, and Lys110), was found to be protected from chemical modification rather inefficiently by the 30S ribosomal subunits and not at all by "naked" RNA (Lammi et al. 1982). Thus, loop L7 is either involved in a protein–protein interaction with the ribosome or, as suggested by Lammi et al. (1982), is part of a functionally important molecular hinge.

The third region of C-domain implicated in the interaction with ribosomes consists of most of loop L8 and of strand B9, and comprises Thr163, Lys164, Glu166, Arg168, Gln169, Ile171, Val173, Leu174, and Ala175. All these residues, with the exception of Arg168, which has been identified by site-directed mutagenesis (Petrelli et al., 1998; R. Spurio & C.O. Gualerzi, unpublished results), have been indicated by NMR as being affected by the interaction. (Figs. 4 and 5A,B).

It has been pointed out (Garcia et al., 1995b) that the structure of the C-domain of IF3 resembles that of the human U1A protein. The cocrystal structure of this protein complexed with the stem-loop II of U1 snRNA (Oubridge et al., 1994) and the NMR structure of a complex with another RNA fragment (Allain et al., 1997) have been determined. The interaction of U1A with RNA involves strands B1 and B3 containing the consensus RNPs motifs of U1A and two loops: one connecting strand B1 and helix H1 and the other connecting strand B2 and strand B3. The corresponding regions in IF3 are strands B7 and B9 and the loops L7 and L8 that, as seen above, show interactions with the 30S ribosomes.
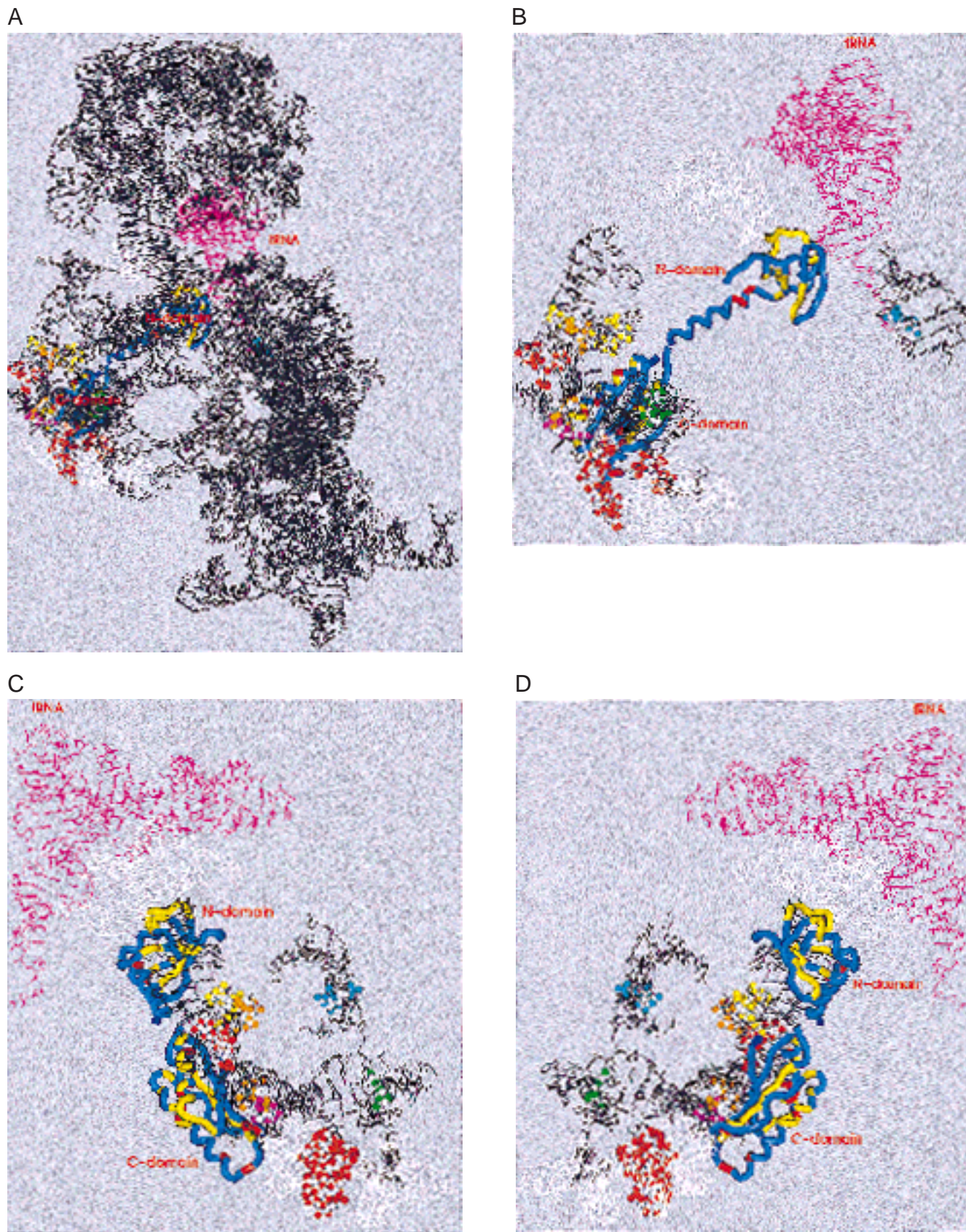
A

B

C

D

FIGURE 6. (*Legend on facing page.*)

Using the DALI Server (Holm & Sander, 1993; http://www2.ebi.ac.uk/dali), we observed additional structural homology between the IF3 C-domain and the enzymes DNaseI (Lahm & Suck, 1991; Weston et al., 1992; PDB code: 3dni), HhaI DNA methyltransferase (Klimasauskas et al., 1994; PDB code: 1hmy), TaqI DNA methyltransferase (Schluckebier et al., 1995; PDB code: 2adm), the G-domain of the elongation factor G (Czworskowski et al. 1994; Aevarsson et al., 1994; PDB code: 1dar), and the N-terminal domain of glutamyl-tRNA synthetase (Nureki et al., 1995; PDB code: 1gln). The similarity in the mode of interaction with nucleic acids make the homologies of IF3 with DNaseI and the HhaI DNA methyltransferase particularly interesting.

For DNaseI (Lahm & Suck, 1991; Weston et al., 1992) three loops have been implicated in interacting with the minor groove of the DNA. The first two loops are equivalent to loops L6 and L7 of IF3, while the third loop is not present in IF3. The residues of the first loop interacting with DNA are Arg9, Glu13, and Thr14 (corresponding to Arg99, Thr102, and Glu104 of IF3), and those of the second loop are Arg41 and Ser43 (corresponding to Phe130 and Ile140 of IF3). As seen above, Arg99, Thr102, and Phe130 have been shown to be involved in binding to 30S by several criteria. The HhaI DNA methyltransferase (Klimasauskas et al., 1994) consists of a large domain, which is structurally homologous to the C-domain of IF3, and a small domain. The DNA is bound in the cleft between these two domains. In the large domain, the loop connecting strand B1 and helix H1 is involved in DNA binding. This loop, though much longer, corresponds to loop L6 of IF3. Other important residues in the binding of the HhaI DNA methyltransferase to the DNA are located in the β-strands B2 and B3 and α-helix H2. The corresponding regions in IF3 (strands B7, B9, and helix H4) show interactions with 30S as well. Additional similarities of the 30S binding site of the IF3 C-domain exist with the nucleotide binding sites of the G-domain of EF-G (Czworskowski et al. 1994; Aevarsson et al. 1994) and of the N-terminal domain of glutamyl-tRNA synthetase (Nureki et al., 1995). In both cases the binding region is located in the loop regions: the loop between strand B2 and helix H2 of EF-G, which corresponds to loop L7 of IF3, and the loop between β-strand B1 and helix H1 of the N-terminal domain of glutamyl-tRNA synthetase, which corresponds to loop L6 of IF3.

Nature of the ribosome binding site of the N-domain

The NMR data obtained in this study and the available amount of biochemical information concerning the interaction of the N-domain with the 30S subunit are definitely less abundant and perhaps not as striking as that obtained for the C-domain. According to the model proposed (Pon et al., 1982b), which seems to be supported by the present data, IF3 binds stably to the ribosome through one of its active sites (located in the C-domain) and establishes a fluctuating interaction with the other site (located in the N-domain). Thus, the 30S-N-domain interaction, though crucial for the biological activity of IF3, plays a role of secondary importance from the thermodynamic point of view; actually, because it should be easily dissociated, it is not surprising that the N-domain–30S interaction makes use of fewer and less extensive contacts than those of the C-domain. Furthermore, unlike with the 30S–C-domain interaction, which is predominantly, if not exclusively, an RNA–protein interaction, there are good reasons to believe that protein–protein interactions play an important role in the ribosomal interaction of the N-domain (Pon et al., 1982a). Nonetheless, a number of amino acid residues have been clearly shown to be affected by the 30S subunits in the NMR spectra (Gln8, Ala10, Asn16, Gly17, Glu18, Gln22, Asp52, Ile56, Cys65, Lys72, Phe73, and Ser78) and to be implicated in the functional interaction of IF3 with the ribosome by other criteria (Lys2, Lys5, Arg6, Cys65, Tyr70, Tyr75, and Lys79).

The N-terminal hexapeptide MKGGKR is the only portion of the N-domain that is known to contribute (directly or indirectly) to the thermodynamic stability of the IF3-30S interaction. It has been shown that proteolytic cleavage of this peptide substantially reduces the affinity for the 30S subunit as well as the biological

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FIGURE 6. Model of the complex of initiation factor IF3 with the 30S ribosomal subunit. **A**: Overview of the N- and C-domains of IF3 docked to the 16S rRNA in the 30S subunit seen from the solvent side. **B**: Close-up of **A** displaying only the relevant features of the model. **C**, **D**: A −908 and a +908 rotation around the vertical axis of the image presented in **B**. In all panels the 3D model of the 16S rRNA within the 30S ribosomal subunit is that based on EM reconstructions as described in Mueller & Brimacombe (1997) and based on the coordinates provided by the same authors specifically. The 16S rRNA is shown (in its entirety only in **A**) completely in black but for the portions to which IF3 has been chemically crosslinked, namely, helices 45 (upper) and 25–26 (lower), which are shown in white. Other nucleotides indicated are those protected by IF3 from kethoxal (yellow) and CMCT (orange). Nucleotide G791, which is partially protected from kethoxal and functionally implicated in IF3 binding by mutagenesis, is indicated in purple. Nucleotides displaying hyperreactivity in the presence of IF3 to DMS (green) and kethoxal (turquoise) or hypersensitivity to RNase V1 (red) are also indicated. Further details are found in the text (see Gualerzi & Pon (1990) for a review of these data). In all panels a P-site bound tRNA is displayed in magenta in the position indicated by Mueller & Brimacombe (1997). IF3 is represented as a blue tube in which the C-terminus of the N-domain and the N-terminus of the C-domain are displayed in darker blue and the residues affected by 30S interaction in yellow (NMR data) or red (mutagenesis or chemical modification data).

activity of IF3 (Lammi et al., 1987). Furthermore, Lys2 and Lys5, which were exposed to modification with pyridoxal phosphate in the absence of 30S ribosomal subunit, became protected in its presence (Ohsawa & Gualerzi, 1981). Because the modification of these lysines did not result in IF3 inactivation, however, Arg6 remains the most likely candidate for the establishment of an interaction with the ribosome. The results of other chemical modifications and mutagenesis also allowed the identification of Cys65, Tyr70, Tyr75, and Lys79 as functionally important, yet perhaps only marginally (or indirectly) involved in binding to the 30S subunits. The -SH group of Cys65 was modified by various reagents, including fluorescent- and spin-labels. These experiments indicated that Cys65 is not essential for the biological activity of IF3 but that its rate of modification is slower in 30S-bound IF3 and a nitroxide spin-label at Cys65 became immobilized in titrations with 30S subunits, but not with nucleic acids such as random poly(A,U,G). It was suggested that Cys65, which is exposed in free IF3, is located at the edge of the IF3 binding site in the 30S-IF3 complex (Pon et al. 1982a). Like Tyr107, which is located in the "primary RNA binding site" of the molecule (see above), both Tyr residues present in the N-domain (positions 70 and 75) were found to be accessible to lactperoxidase-catalyzed iodination in free IF3 and fully protected in 30S-bound IF3. Isolated RNA, on the other hand, was found to protect Tyr70 from modification, but not Tyr75. Furthermore iodination of Tyr70 did not prevent the binding of IF3 to the ribosome, but resulted in the formation of a partially inactive complex, whereas modification of Tyr75 did not produce any detectable inactivation of IF3 in vitro (Bruhns & Gualerzi, 1980). A direct influence of Tyr75 on the activity of IF3 in vivo is clearly suggested, however, by the isolation of three *infC* alleles in which IF3 displays a reduced capacity to discriminate against initiation complexes containing a noncanonical initiation triplet. In all three identified mutants, Tyr75 was found to be replaced by other residues (Sussman et al., 1996). The functional relevance of this part of the IF3 molecule is further indicated by the finding that, in the NMR spectra, the cross peaks belonging to nearby residues (i.e., Lys72, Phe73, and Ser78) are clearly affected by the addition of 30S subunits, and that deletion of Lys79 resulted in more than 50% loss of IF3 activity (Petrelli et al., 1998; R. Spurio & C.O. Gualerzi, unpublished results).

Docking IF3 to its binding site on the 30S ribosomal subunit

Having established that IF3 contains two separate ribosomal binding sites (one in each of its domains) and having just described their molecular facets, for a better understanding of how IF3 might work, we think it relevant to suggest a plausible orientation of this mol-

ecule on the 30S ribosomal subunits. Thus, the sites of the 16S rRNA molecule that have been found to be relevant for the interaction with IF3 have been highlighted with different colors (Fig. 6) in the recently released 3D model of the 30S ribosomal subunit based on electron-microscopy reconstructions (Mueller & Brimacombe, 1997). The main binding site of IF3 on the 30S subunit contains the bulged stem-loop 674–713 (helix 23) and the 783–799 stem-loop (helix 24). In fact, a G-to-A transition at position 791 (purple) increases tenfold the dissociation rate constant of the IF3-30S complex (Tapprich et al., 1989) and modification of the 16S rRNA by the guanosine-specific reagent kethoxal inhibits IF3 binding (Pon & Gualerzi, 1974), whereas G-700, G-703 (yellow), and G-791 (purple) are specifically protected from reaction with this reagent by IF3 (Moazed et al., 1995). Furthermore, two rRNA regions (both white), the main one (819–859, corresponding to helices 25 and 26) within the central domain and the minor one (1506–1529, corresponding to helix 45) in the 3′-end region of the 16S, have been crosslinked to IF3 (Ehresmann et al., 1986). In agreement with these data is the recent finding that an inversion of the sequence (G-A to A-G) at positions 1530/1531 (at the edge of helix 45) results in a tenfold decrease in the affinity of IF3 for the 30S subunit (Firpo et al., 1996). Moreover, this model is consistent with the finding that all 16S rRNA sites, which become hypersensitive or hyperexposed in the presence of IF3 to chemical or enzymatic probes such as RNase V1 (red), DMS (green), and kethoxal (turquoise; Muralikrishna & Wickstrom, 1989; Moazed et al., 1995), are found at the edge or outside the IF3 binding site, confirming the conformational nature of the change leading to the hypersensitivity.

If docking of IF3 to the ribosomal subunit is done with the assumption that the primary rRNA binding site of the C-domain is in contact with the main rRNA sites crosslinked to and/or affected by IF3, which are clustered in the central area of the subunit, the emerging picture is that of a molecule that is implanted through extensive RNA–protein interactions in the central part of the ribosomal particle and reaches the upper margin of the side lobe (platform) where its N-domain touches helix 45 (i.e., the last 3′ terminal helix of 16S rRNA). IF3 is known to act as a fidelity factor that determines the kinetic discrimination against spurious 30S initiation complexes by forcing the dissociation of the noncanonical codon-anticodon interactions at the ribosomal P-site (Gualerzi et al., 1971; Risuleo et al., 1976; Hartz et al., 1989). Theoretically, IF3 could accomplish this function by a direct inspection of the codon-anticodon interaction and/or the anticodon stem-loop of initiator tRNA (Hartz et al. 1990) or affecting the conformation of the 30S ribosomal subunits (Pon & Gualerzi, 1974; Pon et al., 1982b). On the basis of the model presented in Figure 6, one would conclude that IF3 is bound too far

away from both the anticodon stem-loop of the tRNA and the P-decoding site to inspect them directly. A stable interaction of the C-domain with the central part of the 30S subunit and a fluctuating interaction between N-domain and platform could then induce a conformational change around the cleft between the platform and the head and thereby affect the P-site interaction of the tRNA (Fig. 6, magenta) which occurs in this region of the 30S subunit.

MATERIALS AND METHODS

Preparation of biological material

Initiation factor-free 30S ribosomal subunits were prepared from *E. coli* MRE600 cells as previously described (Risuleo et al., 1976). Translation initiation factor IF3 was obtained by overexpressing the *infC* gene cloned in pPLc2833 expression vector transformed into *E. coli* JM109/pcI cells essentially as described (Brombach & Pon, 1987). For the isotopic labeling of IF3, the cells were grown in a minimal medium containing $^{15}NH_4Cl$, as described previously (Sette et al., 1997). Electrophoretically homogeneous ^{15}N-labeled IF3 was purified following the published procedure (Pawlik et al., 1981).

NMR spectroscopic methods

Two-dimensional spectra were recorded at 600 MHz on a Bruker AMXT, and 3D NOESY-($^{15}N,^1H$)-HSQC and 3D TOCSY-($^{15}N,^1H$)-HSQC were recorded at 750 MHz on a Varian Unity Plus, using mixing times of 100 ms and 80 ms for the NOESY and TOCSY spectra, respectively. The titrations with 30S ribosomal subunits were followed by recording 2D ($^{15}N,^1H$)-HSQC spectra. Titrations were performed by direct addition of small aliquots of 30S ribosomal subunits to the NMR tube. The stoichiometric ratio 30S/IF3 was increased in these experiments up to 0.55%. To prevent denaturation and/or inactivation of the 30S ribosomal subunits, the solution contained 20 mM KP_i buffer (pH 6.5), 5 mM $MgSO_4$, 200 mM KCl, and 1 mM dithiothreitol. Throughout all NMR experiments the temperature was maintained at 315 K.

Amide signal intensities were determined by integrating the volumes or measuring the peak heights (in case of partial overlap) of the cross peaks in the 2D ($^{15}N,^1H$)-HSQC spectra observed before and after addition of 30S ribosomal subunits. Because a fixed, small integration area had been used for all the analysis of the titration data, determination of peak heights or volumes gave essentially the same results. The intensity change induced by the ribosomes was determined, for each cross peak, calculating the relative change in intensity obtained in the presence and absence of ribosomes according to the relation $(V - V_0)/V_0$, where V is the intensity measured in the presence of a given amount of 30S subunits and V_0 is the intensity measured in the free protein.

Modeling

The coordinates of the N-domain of *E. coli* IF3 were obtained by replacing the sidechains in the X-ray structure of *B. Stearothermophilus* IF3 (PDB entry: 1TIG; Biou et al., 1995) by those of *E. coli* IF3, followed by energy minimization using X-PLOR, version 3.8 (Brünger, 1992). Ribbon views were generated with the program MOLMOL (Koradi et al., 1996). The coordinates for the 3D model of the 16S rRNA that was used in modelling the IF3-30S complex were those described by Brimacombe & Mueller (1997).

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