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Accelerated Publications

Escherichia coli Initiation Factor 3 Protein Binding to 30S Ribosomal Subunits Alters the Accessibility of Nucleotides within the Conserved Central Region of 16S rRNA†

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Received July 5, 1989; Revised Manuscript Received July 26, 1989

ABSTRACT: Translational initiation factor 3 (IF3) is an RNA helix destabilizing protein which interacts with strongly conserved sequences in 16S rRNA, one at the 3′ terminus and one in the central domain. It was therefore of interest to identify particular residues whose exposure changes upon IF3 binding. Chemical and enzymatic probing of central domain nucleotides of 16S rRNA in 30S ribosomal subunits was carried out in the presence and absence of IF3. Bases were probed with dimethyl sulfate (DMS), at A(N-1), C(N-3), and G(N-7), and with *N*-cyclohexyl-*N*′-[2-(*N*-methyl-4-morpholinio)ethyl]carbodiimide *p*-toluenesulfonate (CMCT), at G(N-1) and U(N-3). RNase T1 and nuclease S1 were used to probe unpaired nucleotides, and RNase V1 was used to monitor base-paired or stacked nucleotides. 30S subunits in physiological buffers were probed in the presence and absence of IF3. The sites of cleavage and modification were detected by primer extension. IF3 binding to 30S subunits was found to reduce the chemical reactivity and enzymatic accessibility of some sites and to enhance attack at other sites in the conserved central domain of 16S rRNA, residues 690–850. IF3 decreased CMCT attack at U701 and U793 and V1 attack at G722, G737, and C764; IF3 enhanced DMS attack at A814 and V1 attack at U697, G833, G847, and G849. Many of these central domain sites are strongly conserved and with the conserved 3′-terminal site define a binding domain for IF3 which correlates with a predicted cleft in two independent models of the 30S ribosomal subunit.

Escherichia coli IF3[1] is a basic polypeptide of 180 residues, 20 520 g/mol (Sacerdot et al., 1982), which catalyzes efficient translation of mRNA bound to 30S ribosomal subunits (Gualerzi et al., 1986; Calogero et al., 1988). Under physiological ionic conditions, IF3 binds to free 30S ribosomal subunits, inhibiting their association with 50S ribosomal subunits to form 70S ribosomes (Goss et al., 1982), unless both mRNA and fMet-tRNA have bound to form an initiation complex.

Extensive studies of the structure of 30S ribosomal subunits, 16S rRNA, and the 30S ribosomal proteins have led to consensus models for the secondary structure of 16S rRNA and the sites of RNA–protein interactions within the subunit (Brimacombe, 1988; Stern et al., 1989). However, the functional roles of most parts of the 30S subunit remain unknown. In particular, the structural transitions which IF3

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[1] Abbreviations: CMCT, *N*-cyclohexyl-*N*′-[2-(*N*-methyl-4-morpholinio)ethyl]carbodiimide *p*-toluenesulfonate; DMS, dimethyl sulfate; DTT, dithiothreitol; EDTA, (ethylenedinitrilo)tetraacetate; IF, initiation factor; RNase, ribonuclease; $NaDodSO_4$, sodium dodecyl sulfate.

0006-2960/89/0428-7505$01.50/0

induces in 30S ribosomal subunits to activate translation are not understood. IF3 binds to 30S ribosomal subunits in the cleft between the head and the platform, in close proximity to the 3′ end of 16S rRNA on the platform (Oakes et al., 1986; Stöffler & Stöffler-Meilecke, 1986).

The interactions of IF3 with synthetic oligonucleotides and polynucleotides (Wickstrom et al., 1980; Schleich et al., 1980; Wickstrom, 1981) and with the 3′-terminal 49 nucleotides of *E. coli* 16S rRNA (Wickstrom, 1983; Wickstrom et al., 1986) revealed that IF3 is an RNA helix destabilizing protein, which binds to the most strongly conserved nucleotides near the 3′ end of 16S rRNA. The interaction is primarily non-ionic and sequence specific. In contrast, ribosomal protein S1 was found unable to protect the same 16S rRNA fragment at physiological ionic strength (Wickstrom, 1986).

Cross-linking (Ehresmann et al., 1986), antisense oligomer binding (Firpo & Hill, 1987), and site-directed mutagenesis (Tapprich et al., 1989) revealed that IF3 also binds to a site in the middle of 16S rRNA. The sequence at the 3′-terminal site is virtually conserved in all forms of life, from bacteria to humans, in both mitochondrial and cytoplasmic ribosomes (van Knippenberg et al., 1984), and the site in the middle of 16S rRNA includes portions which are also strongly conserved (Woese et al., 1983).

In order to elucidate the impact of IF3 binding on the structure of 16S rRNA in the central domain, 30S ribosomal subunits were probed chemically and enzymatically in the presence and absence of IF3. Bases accessible to solvent were probed chemically with DMS, at A(N-1), C(N-3), and G(N-7), and with CMCT, at G(N-1) and U(N-3). RNase T1 and nuclease S1 were used to probe unpaired nucleotides, and RNase V1 was used to monitor base-paired or stacked nucleotides. The sites of attack were identified by primer extension (Moazed et al., 1986; Mougel et al., 1987). IF3 binding to 30S subunits was found to reduce the chemical reactivity and enzymatic accessibility of some sites and to enhance attack at other sites in the conserved central domain of 16S rRNA, nt 690–850. Many of these nucleotides are conserved in mammalian rRNA, underlining the probable importance of this protein–RNA interaction for the function of all ribosomes.

Materials and Methods

Buffers. TKM buffer: 10 mM Tris-HCl (pH 7.4), 50 mM KCl, 10 mM $Mg(OAc)_2$, and 7 mM 2-HSEtOH. TNM buffer: 50 mM Tris-HCl (pH 7.4), 100 mM NH_4Cl, 1 mM $MgCl_2$, and 6 mM 2-HSEtOH. CMK buffer: 50 mM sodium cacodylate (pH 7.2), 50 mM KCl, 20 mM $Mg(OAc)_2$, and 1 mM DTT. BMK buffer: 50 mM K_3BO_3 (pH 8.1), 50 mM KCl, 20 mM $Mg(OAc)_2$, and 0.5 mM DTT. RT buffer: 50 mM Tris-HCl (pH 8.3), 60 mM NaCl, and 10 mM DTT. TBE buffer: 89 mM Tris–H_3BO_3 (pH 8.3) and 8 mM EDTA.

Materials. CMCT and DMS were purchased from Aldrich, RNase T1 was from Sankyo (Japan), nuclease S1 was from Sigma, RNase V1 was from P-L Biochemicals, bacteriophage T4 polynucleotide kinase was from New England Biolabs, avian myeloblastosis virus reverse transcriptase was from Molecular Genetic Resources (Tampa, FL), and DNase I and placental RNase inhibitor were from Boehringer Mannheim Biochemicals. [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from Du Pont/New England Nuclear.

Purification of IF3 and Ribosomal Protein S1. IF3 was purified from the high-expression strain *E. coli* BL21-(DE3)[pMKP23] as described (Muralikrishna & Wickstrom, 1989; Wickstrom & Laing, 1988). Recombinant IF3 from this strain is expressed and isolated almost exclusively as the long form; the short form is clearly an artifact of purification. Ribosomal protein S1 was isolated from ribosomes of *E. coli* MRE600 as described (Muralikrishna & Suryanarayana, 1987). Protein was estimated according to Bradford (1976).

Preparation of 30S Subunits and 16S rRNA. All steps were carried out essentially as described by Moazed et al. (1986), at 4 °C unless indicated otherwise. *E. coli* MRE600 cells (15 g) were ground with 30 g of alumina and extracted with TKM buffer containing 0.5 unit of DNase I/mL. After sedimentation for 30 min at 30000*g*, the upper three-fourths of the supernatant was sedimented at 45000 rpm for 4 h in a Beckman 70Ti rotor. The ribosomal pellet was dissolved in TKM buffer containing 1 M NH_4Cl, left on ice overnight, and sedimented as above to pellet ribosomes. The 70S pellet was resuspended in TNM buffer at a concentration of 600 A_{260} units/mL and dialyzed against 600 volumes of this buffer for 6 h with two changes. The dissociated subunits were layered on 13.0-mL, 10%–40% (w/v) sucrose gradients in TNM buffer and sedimented for 17 h at 26000 rpm in an SW40 rotor. Fractions corresponding to the 30S peak were pooled and diluted with an equal volume of TNM buffer. The $MgCl_2$ concentration was increased to 10 mM, and subunits were pelleted at 30000 rpm in an SW40Ti rotor for 18 h. The 30S pellet was resuspended in 500 μL of 10 mM Tris-HCl (pH 7.6), 60 mM NH_4Cl, 10 mM $Mg(OAc)_2$, and 6 mM 2-HSEtOH and stored in a small portions at −80 °C. The 30S subunits were free from IF3, as assayed by immuno double diffusion (Howe et al., 1978). Samples of 30S subunits were activated by incubation at 42 °C for 25 min as described (Moazed et al., 1986), prior to chemical modification or nuclease hydrolysis.

Naked 16S rRNA was prepared from activated 30S subunits by three extractions with phenol and two extractions with $CHCl_3$ performed in the presence of 0.5% $NaDodSO_4$ and 3 mM EDTA. The 16S rRNA was precipitated by the addition of 2.5 volumes of 95% EtOH in the presence of 0.3 M NaOAc, sedimented, washed with 75% EtOH, and dried under vacuum. The final 16S rRNA pellet was dissolved in 1 mM EDTA (pH 7.0).

Preparation of RNA Transcripts. Plasmid pMKP23, containing the *infC* gene (Muralikrishna & Wickstrom, 1989), was linearized by *Stu*I hydrolysis, and pAP3, containing the α-operon leader (Deckman & Draper, 1985), was linearized by *Hin*dIII hydrolysis. The linearized plasmids were transcribed with bacteriophage T7 RNA polymerase essentially as described (Deckman & Draper, 1985). T7 RNA polymerase was purified from the overproducing strain BL21-(DE3)[pAR1219] as described by Grodberg and Dunn (1988). The transcription mixture contained 100 mM Tris-HCl (pH 7.6), 8 mM $MgCl_2$, 8 mM DTT, 1 mM each of ATP, GTP, CTP, and UTP, 60 units of placental RNase inhibitor, 15 μg of purified DNA, and 4.5 μg of T7 RNA polymerase in a total volume of 300 μL. After incubation at 37 °C for 1.5 h, the template DNA was digested with 4 μL of DNase I (23 units/μL) at 37 °C for 30 min; the RNA transcript was extracted twice with phenol and twice with $CHCl_3$. The yield of RNA was about 400 μg.

Enzymatic Hydrolysis and Chemical Modification. Each assay contained about 23 pmol (corresponding to 12.5 μg of 16S rRNA) of heat-activated 30S subunits, or 12.5 μg of RNA transcript, in 20 μL of the appropriate buffer. Before treatment withh an enzyme or a chemical reagent, a 5- or 10-fold molar excess of IF3 was added, and the mixture was incubated at 37 °C for 5 min and then left on ice for 10 min. Modification reactions were carried out at 4, 21, or 37 °C. For each

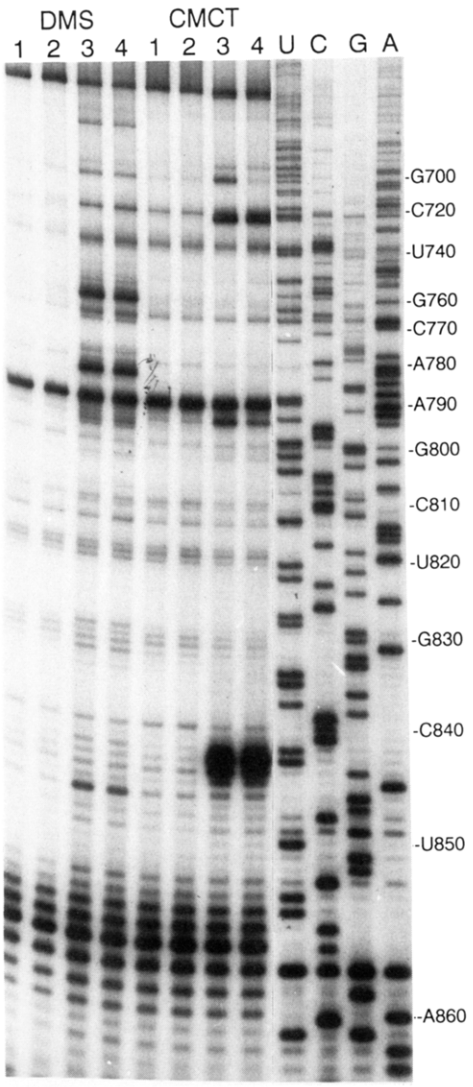

FIGURE 1: Autoradiogram showing altered chemical reactivities of residues in 16S rRNA induced by IF3 binding to 30S subunits. DMS, 6 min at 21 °C; CMCT, 30 min at 21 °C. The order of samples for each reagent is as follows: lane 1, no IF3 and no reagent; lane 2, treated with IF3 and no reagent; lane 3, treated with reagent in the absence of IF3; lane 4, treated with reagent in the presence of a 10-fold molar excess of IF3 relative to 30S subunits. Lanes U, C, G, and A are sequencing products generated in the presence of ddATP, ddGTP, ddCTP, and ddTTP, respectively.

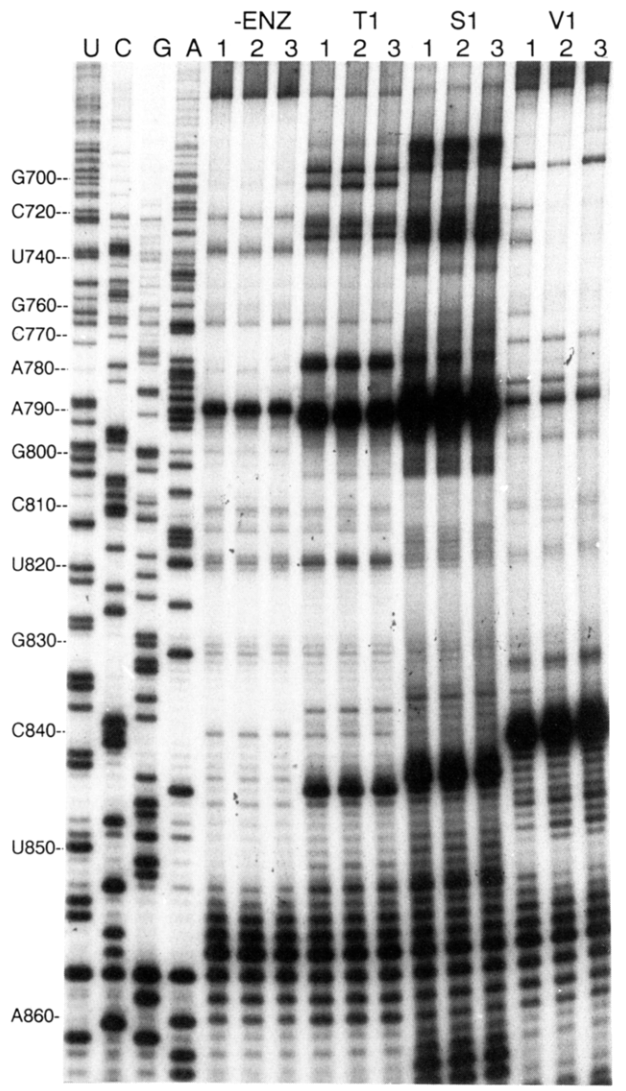

FIGURE 2: Autoradiogram showing altered enzymatic reactivities of residues in 16S rRNA induced by IF3 binding to 30S subunits. −ENZ, treated with no enzyme; T1, RNase T1 (0.015 unit, 15 min); S1, nuclease S1 (75 units, 15 min); V1, RNase V1 (0.2 unit, 15 min). The order of samples for each reagent is as follows: lane 1, no IF3; lane 2, IF3 at a 5-fold molar excess relative to 30S subunits; lane 3, IF3 at a 10-fold molar excess relative to 30S subunits. Lanes U, C, G, and A are sequencing products generated in the presence of ddATP, ddGTP, ddCTP, and ddTTP, respectively.

reaction, a control was treated in parallel, the enzyme or chemical reagent being omitted.

Enzymatic digestions were carried out with 0.015 unit of RNase T1, 75 units of nuclease S1, or 0.2 unit of RNase V1 in 20 μL of CMK buffer. After incubation for 15 min, 100 μL of 0.3 M NaOAc (pH 6.0), 0.5% $NaDodSO_4$, and 6 mM EDTA was added, and 16S rRNA was extracted three times with phenol and two times with $CHCl_3$ and then precipitated twice with EtOH (as described for the naked 16S rRNA above). The deproteinized 16S rRNA pellet was finally dissolved at 1 pmol/μL in 1 mM EDTA (pH 7.0).

Chemical modification reactions were diluted to 150 μL before the reagents were added. DMS modification was carried out in CMK buffer in the presence of 0.5 μL of DMS for 6 min, DMS was inactivated by the addition of 60 μL of 1 M 2-HSEtOH, and the reaction was left on ice for 10 min. CMCT modification was carried out in BMK buffer in the presence of 50 μL of CMCT (42 mg/mL in BMK buffer) for 30 min. All reactions were stopped by precipitation with 100 μL of 0.3 M NaOAc and 650 μL of 95% EtOH at −80 °C for 30 min and sedimented at 14000*g* for 15 min. The pellets were washed with 0.5 mL of 75% EtOH, dried under vacuum, resuspended in 200 μL of 0.3 M NaOAc, 0.5% $NaDodSO_4$, and 6 mM EDTA, and extracted with phenol and $CHCl_3$ as above.

Primer Extension. The primers 5′-d(T-A-A-G-G-A-G-G-T-G), complementary to residues 1542–1533 of 16S rRNA (Gutell et al., 1985), 5′-d(C-C-G-T-A-C-T-C-C-C-C-A-G-G-C-G-G), complementary to residues 895–879 of 16S rRNA, 5′-d(G-G-C-G-C-G-A-A-T-T-T-C-G-C-C-A-T-T), complementary to residues 330–313 of *infC* (Sacerdot et al., 1982), and 5′-d(C-G-A-A-G-T-T-A-A-T-G-C-G-A-T-T-A-C), complementary to residues 158–140 of the α-operon leader (Deckman & Draper, 1985), were prepared with a Biosearch 8750 DNA synthesizer using the phosphoramidite method and purified by reversed-phase chromatography (Wickstrom et al., 1988). An aliquot of 20 pmol of each synthetic oligodeoxynucleotide primer was labeled at its 5′ end with ^{32}P according to the method of Hartz et al. (1988). The hybridization mixture, containing about 0.6 pmol of modified or unmodified

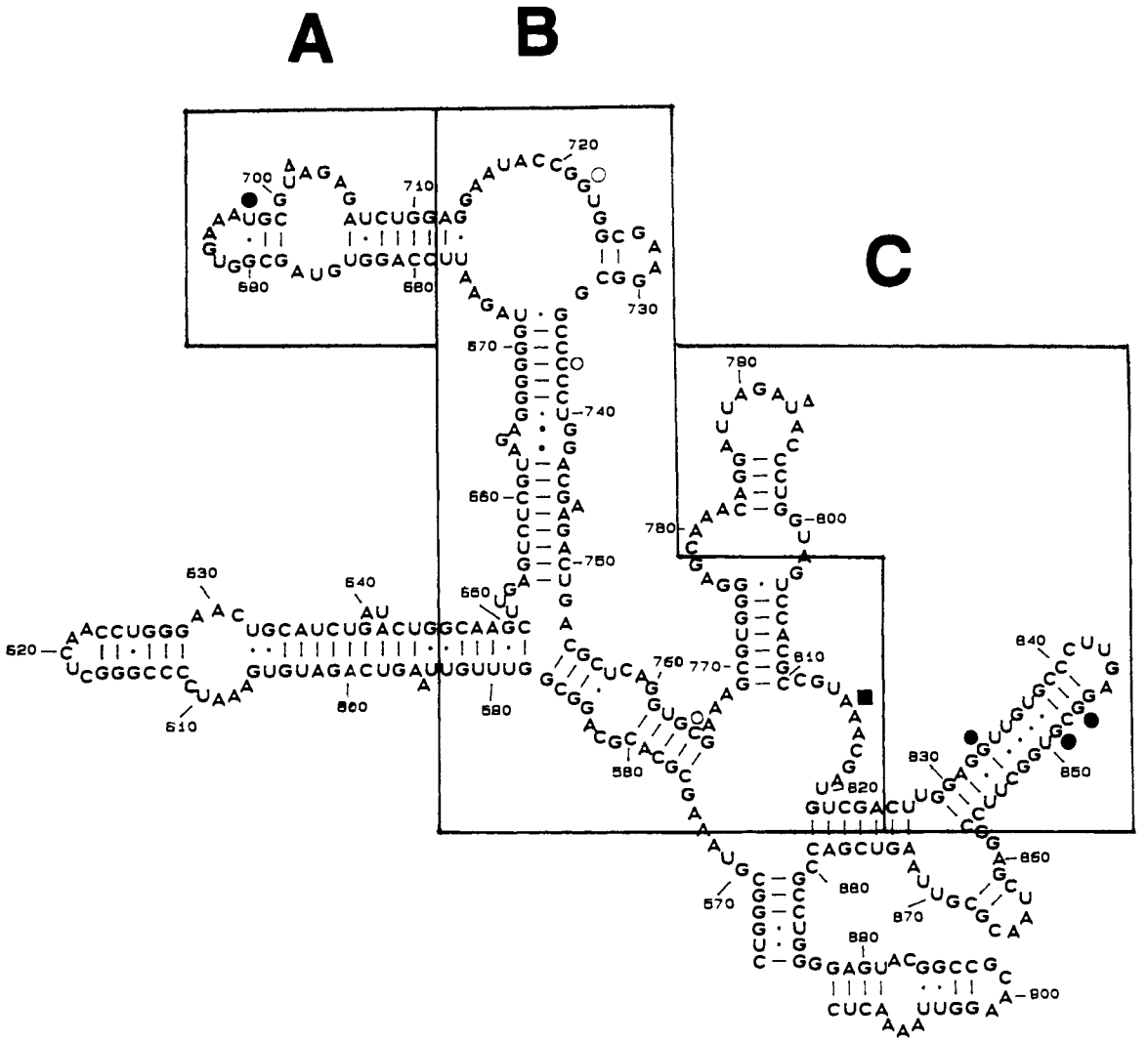

FIGURE 3: Secondary structure of the central region of 16S RNA [adapted from Gutell et al. (1985)] showing sites that are altered in the presence of IF3 in 30S subunits. (○) RNase V1 attack reduced by IF3; (●) RNase V1 attack enhanced by IF3; (■) DMS attack enhanced by IF3; (△) CMCT attack reduced by IF3.

RNA and 0.6 pmol of the 5′ end labeled primer, was incubated at 65 °C for 3 min in 4 μL of RT buffer. Each reaction was then chilled immediately in a dry ice–EtOH bath for 1 min and then allowed to thaw on ice. The annealing mixtures were sedimented briefly. Primer extension was done in 8 μL of RT buffer containing 10 mM $Mg(OAc)_2$ at 47 °C for 30 min in the presence of 1 unit of reverse transcriptase and 360 μM each of dATP, dGTP, dCTP, and dUTP. Sequencing reactions with naked 16S rRNA also contained a 190 μM concentration of the appropriate ddNTP. The reactions were stopped by addition of 16 μL of loading buffer [deionized formamide, 0.02% (w/v) each of xylene cyanol and bromophenol blue], heated to 95 °C for 3 min, and then quickly chilled on ice. Aliquots (6 μL) of the reactions were analyzed on 45 × 33 × 0.04 cm gels containing 8% acrylamide (20:1) and 7 M urea, in TBE buffer; electrophoresis was carried out at 40–45 W for 3–4 h.

Results

The aim of this investigation was to identify changes in reactivities or accessibilities of nucleotides in 16S rRNA toward chemical and enzymatic probes which were induced by the binding of IF3 to 30S ribosomal subunits. Probing experiments were repeated several times at different temperatures and probe concentrations.

An autoradiogram of a chemical probe carried out at 21 °C appears in Figure 1, and the corresponding enzymatic probe results appear in Figure 2. Under the experimental conditions, IF3 should be bound to greater than 99% of the 30S subunits, assuming the reported association constant of 3×10^7 M^{-1} in a very similar buffer (Goss et al., 1982). The autoradiograms are representative of three independent repetitions of the experiments at 21 °C and could be read with confidence through residues 690–850. The strong stop in the 3′-terminal hairpin prevented analysis of that region by primer extension. The chemical and enzymatic results of the 21 °C experiments are summarized schematically in Figure 3 on a secondary structure map of nt 564–912 of 16S rRNA, adapted from Moazed et al. (1986).

Under these conditions, IF3 decreased CMCT attack at U701 and U793 and V1 attack at G722, C737, and C764; IF3 enhanced DMS attack at A814 and V1 attack at U697, G833, G847, and G849. No differences in nuclease S1 or RNase T1 attack could be detected. The small decrease in V1 attack at U697 in the presence of 5-fold IF3 is an artifact in this particular gel; in all other cases V1 attack increased with both 5- and 10-fold IF3. Similarly, the decrease in V1 attack at C853 seen on this gel was not generally repeatable. At 37 °C, the same pattern of protection or exposure was observed as at 21 °C; however, at 4 °C, enhanced DMS attack at A814 was not observed nor was enhanced V1 attack at G833, G847, and G849 or protection from V1 attack at C764 (not shown).

In contrast, ribosomal protein S1 was found unable to protect or expose any site in the central domain of 16S rRNA (not shown). Hence, it is clear that ribosomal protein S1 does not bind to the same rRNA site as IF3. Similarly, probing of IF3 binding to the ribosome binding site regions of pAP3/*Hin*dIII and pMKP23/*Stu*I did not display any sign of structural changes induced upon addition of IF3 (not shown). The transcript sequences included the first 213 nt of *infC* from pMKP23 and the first 245 nt of the α-operon leader from pAP3. It thus appears that although IF3 binds very closely to the site where mRNA initiation regions also bind on 30S subunits, IF3 does not interact detectably with mRNAs, including its own.

Discussion

The pattern of protection or exposure to modifying agents which is induced in the central region of 16S rRNA by IF3 binding to 30S subunits reveals three domains (Figure 3). The sites of increased chemical attack or decreased RNase V1 attack imply sites of increased solvent accessibility and structural destabilization, while sites of decreased chemical attack or increased RNase V1 attack imply regions of sequestration and helix stabilization. The sites of enhanced RNase V1 attack at U697 and decreased CMCT attack at U701 identify an upstream region (A) of sequestration or structural stabilization. On the other hand, decreased RNase V1 attack at G722, C737, and C764 and enhanced DMS attack at A814 delineate an inner domain (B) of helix destabilization and greater nucleotide exposure induced by IF3. Finally, the site of decreased CMCT attack at U793 in the central hairpin loop and the sites of enhanced RNase V1 attack at G833, G847, and G849 identify the existence of a downstream locus (C) of sequestration and helix stabilization.

The latter result agrees with cross-linking evidence for IF3 interaction with residues 819–859 (Ehresmann et al., 1986). Similarly, IF3 protection of U793 is consistent with antisense oligodeoxynucleotide inhibition of IF3 binding to the conserved central loop by an antisense oligomer targeted against residues 787–795 (Firpo & Hill, 1987). Furthermore, oligodeoxynucleotide site-directed mutagenesis of G791 to A791 in that same conserved loop also inhibited IF3 binding (Tapprich et al., 1989). The influence of IF3 on the accessibility of residues U697–C764, domains A and B, constitutes a novel observation. It is noteworthy that the sites of interaction in domains B and C are largely conserved, even in mammalian 18S rRNA (Rairkar et al., 1988).

The interaction of IF3 with both the 3′ terminus and the central domain of 16S rRNA is consistent with three separate models for the rRNA structure and protein distribution of the 30S subunit (Brimacombe, 1988; Stern et al., 1989; Oakes & Lake, submitted for publication). In all cases, the relevant rRNA stems occur in close juxtaposition, separated by a cleft of appropriate size for IF3. This potential IF3 binding cleft is lined with ribosomal proteins previously shown to cross-link with IF3 (MacKeen et al., 1980; Cooperman et al., 1981).

The hydrodynamic data of Gualerzi et al. (1986) imply that IF3 is an ellipsoid with a long axis of about 6.5 nm and a short axis of about 2 nm. Thus, the small size of IF3 probably rules out direct IF3 contacts with each of the nucleotides in the central and 3′ domains whose accessibility is perturbed by IF3 binding to 30S subunits. One may speculate that sites of reduced accessibility or helix stabilization are more likely to represent direct sequestration of RNA residues and that sites of increased accessibility and helix destabilization may represent indirect results of stress on rRNA structure due to IF3 binding elsewhere. Hence, in Figure 3, one may imagine that domains A and C are candidates for direct binding to IF3 and that domain B includes residues which may not bind directly to IF3.

In order to understand the actual mechanism by which IF3 accelerates the rate-limiting step of initiation in the translation of mRNAs bound to 30S subunits, it would be useful to identify which nucleotides of 16S rRNA interact directly with particular amino acids of IF3. Hartz et al. (1989) have recently observed by toeprinting that IF3 inspects the accuracy of the initiator codon–anticodon interaction. This may imply that IF3 also interacts directly with AUG as observed previously (Wickstrom, 1974). In view of the evolutionary conservation of those residues identified above which are affected by IF3 binding, elucidation of the mechanism of catalysis by IF3 would probably add significantly to the understanding of translational initiation in higher organisms.

Acknowledgments

We thank Dr. William Studier and Dr. David Draper for gifts of bacterial strains and plasmids, Dr. Harry Noller for preprints of primer extension protocols, Dr. Albert Dahlberg, Dr. Dixie Goss, and Dr. Larry Gold for communicating results prior to publication, and Adam Riker for excellent technical assistance.

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Stereospecific Nuclear Magnetic Resonance Assignments of the Methyl Groups of Valine and Leucine in the DNA-Binding Domain of the 434 Repressor by Biosynthetically Directed Fractional ^{13}C Labeling†

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Received June 14, 1989; Revised Manuscript Received July 11, 1989

ABSTRACT: Stereospecific ^{1}H and ^{13}C NMR assignments were made for the two diastereotopic methyl groups of the 14 valyl and leucyl residues in the DNA-binding domain 1-69 of the 434 repressor. These results were obtained with a novel method, biosynthetically directed fractional ^{13}C labeling, which should be quite widely applicable for peptides and proteins. The method is based on the use of a mixture of fully ^{13}C-labeled and unlabeled glucose as the sole carbon source for the biosynthetic production of the protein studied, knowledge of the independently established stereoselectivity of the pathways for valine and leucine biosynthesis, and analysis of the distribution of ^{13}C labels in the valyl and leucyl residues of the product by two-dimensional heteronuclear NMR correlation experiments. Experience gained with the present project and a previous application of the same principles with the cyclic polypeptide cyclosporin A provides a basis for the selection of the optimal NMR experiments to be used in conjunction with biosynthetic fractional ^{13}C labeling of proteins and peptides.

Nuclear magnetic resonance (NMR)[1] spectroscopy in solution is by now quite well established as a method for the determination of the three-dimensional structure of proteins [for recent reviews see, for example, Wemmer and Reid (1985), Clore and Gronenborn (1987), Kaptein et al. (1988), and Wüthrich (1989a,b)], and there is keen interest in additional refinements of the method to further improve the precision of the structure determinations. One avenue toward this goal is the use of stereospecific assignments for diastereotopic groups of protons (Wüthrich, 1986; Kline et al., 1988; Driscoll et al., 1989; Güntert et al., 1989), which are not obtained by the generally used sequential resonance assignment procedure for proteins (Wüthrich et al., 1982; Billeter et al., 1982; Wagner & Wüthrich, 1982; Wider et al., 1982). For structure determinations without stereospecific assignments, a set of pseudoatoms replacing the diastereotopic hydrogen atoms was introduced (Wüthrich et al., 1983). This is inevitably a compromise, since the use of these pseudoatoms reduces the precision of the experimental conformational constraints (Wüthrich, 1986). More recently, systematic manual and automated procedures were introduced for obtaining stereospecific assignments for β-methylene groups (Arseniev et al., 1988; Güntert et al., 1989; Hyberts et al., 1987; Wagner et al., 1987; Weber et al., 1988). For more peripheral side-

† Financial support was obtained from the Schweizerischer Nationalfonds (Project Nr. 31.25174.88).
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[1] Abbreviations: NMR, nuclear magnetic resonance; COSY, two-dimensional correlated spectroscopy; TOCSY, two-dimensional total correlation spectroscopy; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid; biosynthetic fractional ^{13}C labeling, biosynthetically directed fractional ^{13}C labeling.

0006-2960/89/0428-7510$01.50/0