Circular Dichroism and 500-MHz Proton Magnetic Resonance Studies of the Interaction of *Escherichia coli* Translational Initiation Factor 3 Protein with the 16S Ribosomal RNA 3′ Cloacin Fragment†

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ABSTRACT: The RNA helix destabilizing properties of *Escherichia coli* initiation factor 3 protein (IF3), and its affinity for an evolutionarily conserved sequence at the 3′ end of 16S rRNA, led us to examine the details of the protein–nucleic acid interactions upon IF3 binding to the 49-nucleotide 3′-terminal cloacin DF13 fragment of 16S rRNA by studying the circular dichroism (CD) and proton magnetic resonance spectra of the RNA, the protein, and their complex. In a physiological tris(hydroxymethyl)aminomethane buffer, where the interaction is primarily nonionic and sequence specific, addition of IF3 decreases the RNA 268-nm CD peak hyperbolically by 19% to an end point of about one IF3 per RNA strand. The titration curve is best fit by an association constant of $(1.80 \pm 0.05) \times 10^7$ M^{-1}, within the range estimated by a nuclease mapping study of the same system [Wickstrom, E. (1983) *Nucleic Acids Res. 11*, 2035–2052]. In a low-salt phosphate buffer without Mg^{2+}, where the interaction is primarily ionic and nonspecific, titration with IF3 decreases the peak CD sigmoidally by 35% to an end point of two IF3s per strand. The titration curve is best fit by an intrinsic association constant of $(1.7 \pm 0.7) \times 10^6$ M^{-1} for each IF3 and a cooperativity constant of 33 ± 6. In a physiological phosphate buffer lacking Mg^{2+}, the dispersion of aromatic proton magnetic resonance peaks and upfield-shifted methyl proton resonances indicates a high degree of secondary and tertiary structure in the protein. In an equimolar mixture of IF3 and RNA cloacin fragment, several changes in identifiable IF3 and RNA resonances are observed. In the aromatic region, resonances of Tyr-107, which is part of the ribosomal binding site of IF3, exhibit a shift or broadening, while resonances of the nonessential Tyr-75 are unaffected. The imino proton resonance of G22-C27 at the top of the cloacin fragment hairpin stem shifts upfield underneath the G18-C31 peak, and the imino proton resonances of the adjacent A20-U29 and U19-G30 base pairs in the center of the hairpin broaden extensively, implicating these base pairs in IF3/RNA complex formation. Both effects were seen previously upon heating the RNA in the absence of protein [Heus, H. A., van Kimmenade, J. M. A., van Knippenberg, P. H., Haasnoot, C. A. G., de Bruin, S. H., & Hilbers, C. W. (1983) *J. Mol. Biol. 170*, 939–956]. The broadening was maintained in the presence of 1 M NaCl, suggesting that this particular interaction may not be ionic. The CD and NMR results may be correlated with nuclease and chemical probe results to yield a model wherein IF3 interacts with the most highly conserved nucleotides of the cloacin fragment.

The *Escherichia coli* translational initiation factor 3 protein (IF3) stimulates the binding of natural and synthetic mRNAs to 30S ribosomal subunits (Wahba et al., 1969; Grunberg-Manago, 1980; Gualerzi & Pon, 1981; Wittmann, 1983). IF3 also accelerates the association and dissociation rates of aminoacyl-tRNAs and initiator tRNA to 30S ribosomal subunits (Gualerzi & Pon, 1981; Gualerzi et al., 1986; Berkhout et al., 1986) and promotes the dissociation of 70S ribosomes into 50S and 30S ribosomal subunits (Chaires et al., 1981; Goss et al., 1982). It has been proposed that IF3 may denature mRNA ribosome binding sites to single strands (Wickstrom, 1974), in order to catalyze base pairing between the ribosome binding site and the 3′ end of 16S rRNA (Shine & Dalgarno, 1974). Such a function has also been proposed for IF3 and ribosomal protein S1 acting in conjunction (van Dieijen et al., 1976). On the other hand, it is just as plausible to propose that IF3 alters the 30S ribosomal subunit conformation so as to favor mRNA·rRNA base pairing and initiator tRNA binding, without direct IF3/mRNA interaction (Gualerzi & Pon, 1981; Pon et al., 1982).

Studies of the protein–nucleic acid interactions of IF3 indicate that IF3 is an RNA helix destabilizing protein with no apparent sequence specificity at low ionic strength and an RNA binding site size of 14 ± 1 nucleotides (Wickstrom et al., 1980; Schleich et al., 1980; Wickstrom, 1981). At physiological ionic strength or above, IF3 binds specifically to the initiation codon AUG (Wickstrom, 1974) and to a 13-nucleotide sequence in the 3′-terminal cloacin DF13 fragment of 16S rRNA, nucleotides 1492–1505, 5′-GUCGUAACAAGGU-3′ (Wickstrom, 1983), which is largely conserved in all forms of life (van Knippenberg et al., 1984). The specificity of the latter interaction is underlined by the fact that IF3 protects the cloacin fragment from nucleases even in the presence of a large excess of tRNA; in contrast, ribosomal protein S1 does not protect the cloacin fragment from nucleases under the same conditions (Wickstrom, 1986). At physiological ionic strength or above, IF3 binds very weakly to homopolynucleotides (Wickstrom, 1981; Schmidt et al., 1985).

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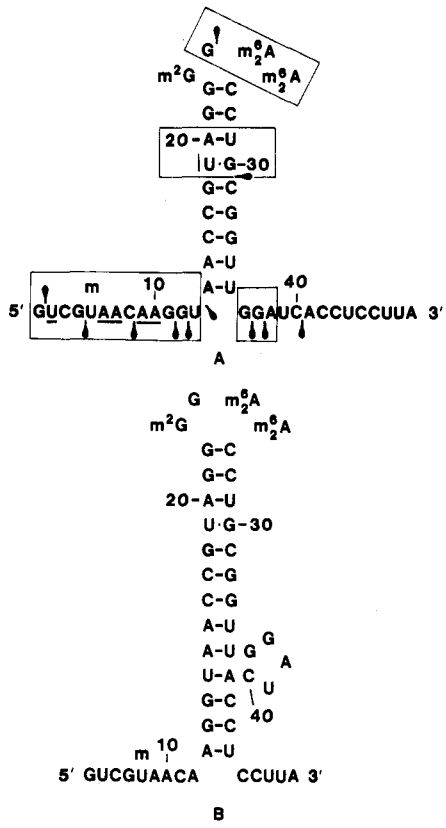

FIGURE 1: Alternative structures of the 49-nucleotide 3′-terminal cloacin DF13 fragment of 16S rRNA, from Wickstrom (1983). Numbering starts at the 5′ end of the fragment, which is residue 1494 of 16S rRNA (Brosius et al., 1978). Boxed regions are evolutionarily conserved (van Knippenberg et al., 1984). Nucleotides protected by IF3 from single-strand-specific nucleases are underlined in structure A; those attacked more readily by single-strand-specific nucleases in the presence of IF3 are indicated by arrows.

Protein–protein cross-linking, RNA–protein cross-linking, and immunoelectron microscopy studies indicate that IF3 binds in the cleft of the 30S ribosomal subunit, bridging from the head of the subunit to the platform, in close proximity to the 3′ end of 16S rRNA on the platform (Gualerzi & Pon, 1981; Chaires et al., 1982; Schwartz et al., 1983; Wittmann, 1983). Thus, IF3 binding to the cloacin fragment correlates with the site of IF3 binding on the 30S ribosomal subunit. The 3′ end of 16S rRNA appears to be essential for IF3 binding, but no 30S ribosomal protein has been identified as essential for IF3 binding (Pon & Gualerzi, 1976; Laughrea et al., 1978).

Optical absorbance vs. temperature (van Charldorp et al., 1981; Heus et al., 1983a), temperature jump (Yuan et al., 1979; Heus et al., 1983b), and 500-MHz proton magnetic resonance (Baan et al., 1977; Heus et al., 1983b) studies of the 16S rRNA 3′ cloacin fragment indicated that Figure 1A is the probable structure of the cloacin fragment in a solution of physiological ionic strength and temperature. On the other hand, nuclease mapping of the cloacin fragment (Wickstrom, 1983; H. A. Heus et al., unpublished results) and chemical probing at a variety of temperatures (H. A. Heus et al., unpublished results) agreed with Figure 1B at lower temperatures. This discrepancy probably means that the extra four base pairs in Figure 1B exchange too rapidly at room temperature to be detected spectroscopically.

Chemical probes of 16S rRNA secondary structure in low-salt-washed 30S ribosomal subunits, which still contain IF3 (Chapman & Noller, 1977; Woese et al., 1980), and nuclease mapping in the presence of IF3 (Wickstrom, 1983) implied that Figure 1A is the probable structure of the cloacin fragment in the presence of IF3. This model correlates with the observation that the octadeoxynucleotide 5′-dAAGGAGGT binds equally well to 70S ribosomes, 30S subunits, and the nonaribonucleotide 5′-ACCUCCUUA, the 3′-terminal nine residues of 16S rRNA (van Duin et al., 1984). The nuclease mapping study implied that IF3 protects some parts of the cloacin fragment but makes other parts more susceptible to nucleases and that the association constant of IF3 with the cloacin fragment under physiological conditions lies within the range $(1.4–7.0) \times 10^7$ M^{-1} (Wickstrom, 1983).

The IF3-cloacin fragment interaction provides a well-defined system for examining RNA helix destabilization upon binding of a sequence-specific protein. Our initial spectroscopic studies, described below, combined with nuclease mapping and chemical probe results, allow us to propose a simple model wherein IF3 interacts with the most highly conserved residues in the cloacin fragment.

Materials and Methods

Preparation of Ribosomes and IF3. Tight-couple 70S ribosomes, prepared from freshly grown *E. coli* MRE 600 cells and washed twice in 1.0 M NH_4Cl (Sobura et al., 1977), were the kind gift of Dr. Albert J. Wahba. The long form of IF3 was prepared and assayed essentially as described by Hershey et al. (1977), with slight modifications and purification to homogeneity by an additional phosphocellulose chromatography step as described (Wickstrom, 1983). IF3 concentrations were estimated by the Coomassie blue assay (Bradford, 1976), $E^{1\%}_{280} = 2.0$ (Hershey et al., 1977), and the molecular mass of IF3, 20 668 daltons (Brauer & Wittmann-Liebold, 1977). Numbering of amino acid residues is based on the nucleotide sequence of the gene (Sacerdot et al., 1982).

Preparation of the 3′-Terminal Cloacin Fragment of 16S rRNA. The 49-nucleotide 3′-terminal fragment of 16S rRNA, residues 1494–1542 (Brosius et al., 1978), was prepared according to Heus et al. (1983b) by using cloacin DF13 (de Graaf & Klaasen-Boor, 1977), the kind gift of Dr. Frits de Graaf. Final purification was done by denaturing gel electrophoresis as described (Wickstrom, 1983). Concentrations were determined spectrophotometrically with an extinction coefficient of 3.8×10^5 M^{-1} cm^{-1} at 260 nm. A nuclease mapping study confirmed the sequence and structure of the fragment (Wickstrom, 1983).

Optical Measurements. Absorption spectra were recorded on an IBM 9420 spectrophotometer. CD spectra were recorded from 320 to 220 nm on a Jasco J-500A spectropolarimeter in a 0.6-mL, 1.0-cm path-length cylindrical cell (NSG-Precision Cells) in a thermostated cell holder, as described previously (Wickstrom, 1981). CD measurements were made at 25 °C on cloacin fragment solutions, to which were added aliquots of 32.8 μM IF3 for subsequent spectra, until well past the saturation point. All measurements were corrected for dilution; CD magnitudes are reported as $\Delta\epsilon$, in units of M^{-1} cm^{-1} per residue. Titration curves of dilution-corrected CD peak magnitudes vs. total IF3 concentrations were fitted to models with the nonlinear least-squares multiparameter fit procedure of the SAS package (SAS Institute), run on an IBM 3081 mainframe. For the hyperbolic CD titration curve, IF3 binding to the cloacin fragment was assumed to be noncooperative; fitting was attempted for both one and two IF3 molecules per RNA. For the sigmoid titration curve, cooperative as well as noncooperative models were tested.

Proton Magnetic Resonance Measurements. Spectra were recorded at 24 °C on a Bruker WM-500 spectrometer equipped with an Aspect 2000 minicomputer, operating in the Fourier-transform mode. Chemical shifts were measured

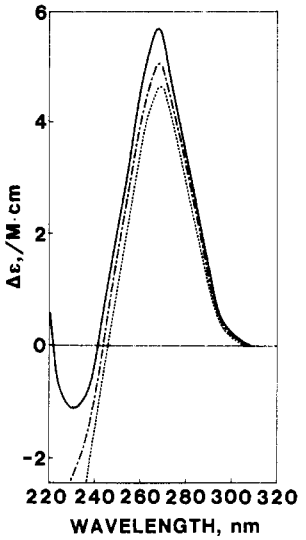

FIGURE 2: Circular dichroism spectra of 0.15 μM 16S rRNA cloacin fragment in 10 mM Tris-HCl, pH 7.4, 100 mM NH_4Cl, 1.0 mM $Mg(OAc)_2$, 1.0 mM dithiothreitol, and 5% glycerol (physiological Tris buffer) at 25 °C, as a function of added IF3. (—) No IF3; (–·–) 0.22 μM IF3; (···) 0.64 μM IF3.

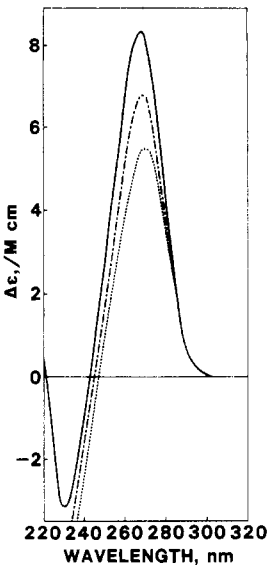

FIGURE 3: Circular dichroism spectra of 0.25 μM 16S rRNA cloacin fragment in 10 mM Na_2HPO_4, pH 7.4, 1.0 mM EDTA, 1.0 mM dithiothreitol, and 5% glycerol (low-salt phosphate buffer) at 25 °C, as a function of added IF3. (—) No IF3; (–·–) 0.41 μM IF3; (···) 0.94 μM IF3.

relative to the solvent water peak and converted to the 2,2-dimethyl-2-silapentane-5-sulfonate standard with temperature and salt calibration curves. To suppress excitation of the strong water peak in samples dissolved in ^1H_2O, a semiselective pulse was used, in combination with an alternate delay acquisition (Haasnoot & Hilbers, 1983). Free induction decays were apodized by an exponential function, causing extra line broadening of 8 Hz. For samples dissolved in ^2H_2O, the residual $^1HO^2H$ signal was presaturated by a gated irradiation (Campbell et al., 1974) of 0.5 s on the $^1HO^2H$ resonance. These spectra were given a Gaussian–Lorentzian transformation for resolution enhancement: GB = 0.15 and LB = 7.

RESULTS

CD Spectra. The CD spectrum of 0.15 μM cloacin fragment in 10 mM $(HOCH_2)_3CNH_2$·HCl, pH 7.4, 100 mM NH_4Cl, 1 mM $Mg(OAc)_2$, 1.0 mM dithiothreitol, and 5% glycerol (physiological Tris buffer) at 25 °C is shown in Figure 2. The spectrum displayed no 295-nm trough, a 268-nm peak with a CD magnitude of 5.7 ± 0.6 M^{-1} cm^{-1}, a 242-nm crossover, and a 231-nm trough with a CD of −1.1 ± 0.2 M^{-1} cm^{-1}. Upon addition of increasing concentrations of IF3, the peak CD magnitude decreased hyperbolically by 19% to an end point of about one IF3 per RNA strand, with a peak red shift of only 2 nm. For comparison, raising the sample temperature to 37 °C decreased the peak CD magnitude by 13%, and at 50 °C the magnitude was reduced by 30%. As seen before (Wickstrom, 1981), IF3 made no significant contribution to the spectrum above 250 nm, but its 230-nm trough dominated the spectrum below 250 nm.

When the CD spectrum of 0.25 μM cloacin fragment was measured in 10 mM Na_2HPO_4, pH 7.4, 1.0 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol, and 5% glycerol (low-salt phosphate buffer) at 25 °C (Figure 3), its shape was virtually the same as that in physiological Tris buffer, but the peak CD was 8.9 ± 0.6 M^{-1} cm^{-1} and the trough CD was −3.1 ± 0.7 M^{-1} cm^{-1}. Titration with IF3 decreased the peak CD sigmoidally by 35% to an end point of two IF3 molecules per strand, with the same 2-nm peak red shift.

Spectra measured in both buffers showed no peak flattening or large red shifts, and no turbidity was observed in the cell. Both observations imply that no aggregation occurred. The absence of a 295-nm trough in the cloacin fragment CD spectra is probably due to the lack or low fraction in the cloacin fragment sequence of double-stranded sequences, which display a 295-nm trough, such as GC, AU, or AC, and the high proportion of AA, GG, and AG sequences, which lack a 295-nm trough; single-stranded regions do not exhibit a 295-nm trough (Gray et al., 1981).

In RNA CD spectra, the near-ultraviolet peak wavelength shifts to the red as the proportion of single-stranded regions increases and that of double-stranded regions decreases. The CD spectrum of fully double-stranded RNA peaks at 260 nm (Gray et al., 1981; Samejima et al., 1968), while the CD spectra of tRNA, rRNA, and mRNA usually peak at 265 nm in solutions similar to physiological Tris buffer, and the CD spectra of single-stranded RNA red shift to 270–275 nm (Brahms & Mommaerts, 1964; Wickstrom, 1971; Blum et al., 1972; Bobst et al., 1974). The occurrence of the peak of the cloacin fragment CD spectrum at 268 nm correlates with the lower fraction of base-paired nucleotides in this RNA than in larger RNAs. The 242-nm crossover and 231-nm trough are further to the blue than is seen in larger RNAs but probably are due to the high G-C content of the cloacin fragment (Gray et al., 1981).

IF3 Titrations of CD Spectra. Titration of the peak CD of the cloacin fragment in physiological Tris buffer by addition of IF3 is shown in Figure 4. The 19% reduction in CD magnitude at the end point correlates by interpolation to raising the temperature to about 41 °C, in the absence of IF3, in which case the extra four base pairs of Figure 1B would be fully melted, the nine base pairs of Figure 1A would still be intact, and single-stranded regions would be less stacked. The titration displayed a hyperbolic decay curve with an apparent end point of one IF3 per RNA. The curve was best fit by assuming that only one IF3 bound per cloacin fragment, yielding an association constant of (1.80 ± 0.05) × 10^7 M^{-1}, corresponding to a free energy of binding of −9.9 ± 0.3 kcal/mol. This result is consistent with a nuclease mapping study of the same system (Wickstrom, 1983), where an equimolar dilution series implied an association constant in the range (1.4–7.0) × 10^7 M^{-1}. A trial assumption of two IF3 molecules per cloacin fragment, binding independently, gave

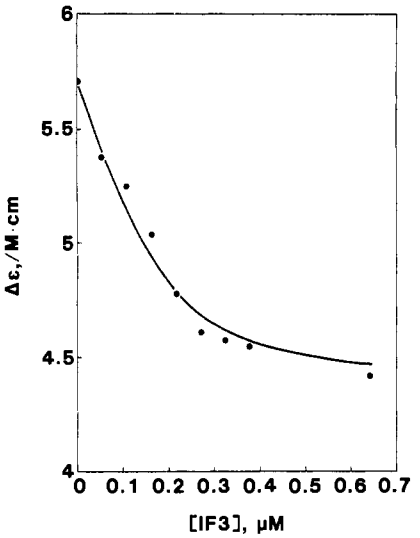

FIGURE 4: IF3 titration of 0.15 μM cloacin fragment RNA circular dichroism in physiological Tris buffer at 268 nm. The smooth curve represents the best fit of the data to a hyperbolic titration curve, which assumes one IF3 bound per RNA, with an association constant of 1.8×10^7 M^{-1}.

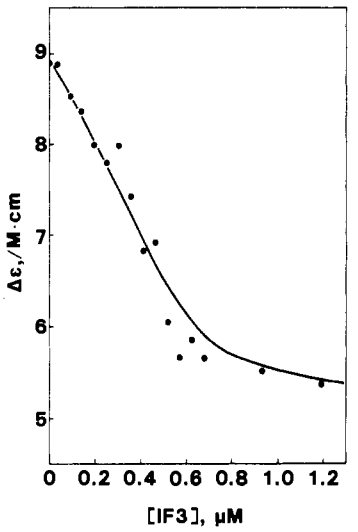

FIGURE 5: IF3 titration of 0.25 μM cloacin fragment RNA circular dichroism in low-salt phosphate buffer at 268 nm. The smooth curve represents the best fit of the data to a sigmoidal titration curve, which assumes two IF3 molecules bound per RNA, with an intrinsic association constant of 1.7×10^6 M^{-1} for each one and a cooperativity constant of 33 for the binding of the second IF3 to an IF3–RNA complex.

a poorer fit, with an association constant for each IF3 of $(8.10 \pm 0.65) \times 10^7$ M^{-1}.

IF3 titration of the peak CD of the cloacin fragment in low-salt phosphate buffer appears in Figure 5. The 35% reduction in CD magnitude, almost twice as much as in physiological Tris buffer, implies complete disruption of secondary structure, as we have seen before for homopolynucleotides in low-salt phosphate buffer (Schleich et al., 1980; Wickstrom, 1981). At this low ionic strength, IF3 binding is not sequence specific, so IF3 molecules cover the entire oligonucleotide, resulting in thorough melting, as opposed to the partial destabilization seen in physiological Tris buffer, where only one IF3 binds per cloacin fragment. The peak CD declined sigmoidally, with an apparent end point of two IF3 molecules per RNA. The titration curve was best fit by assuming independent binding of one IF3 and cooperative binding of a second IF3 to each cloacin fragment. The fitting

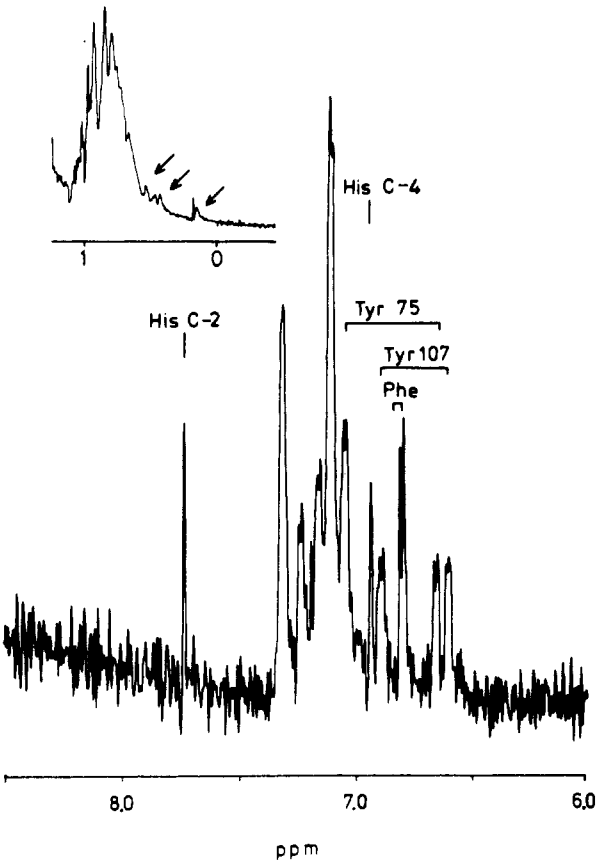

FIGURE 6: 500-MHz proton spectra of 0.2 mM IF3 in ^2H_2O at 24 °C in 25 mM Na_2HPO_4, pH 7.5, 100 mM NaCl, 1.0 mM EDTA, and 1.0 mM dithioerythritol (physiological phosphate buffer). Lower trace shows the aromatic region (312 scans). Assignments are from Paci et al. (1984). Upper trace shows the methyl proton region (3480 scans). Arrows indicate upfield-shifted methyl resonances.

routine yielded an intrinsic association constant of $(1.7 \pm 0.7) \times 10^6$ M^{-1} for each IF3, corresponding to a free-energy change of -8.5 ± 3.5 kcal/mol, and a cooperativity constant of 33 ± 6, corresponding to a free-energy change of -2.1 ± 0.4 kcal/mol. These results agree well with IF3 titration of poly(rA) CD in the same low-salt phosphate buffer, which was fit by an intrinsic association constant of $(1.3 \pm 0.8) \times 10^6$ M^{-1} for each IF3, and a cooperativity constant of 25 ± 7 (Wickstrom, 1981).

NMR Spectra in ^2H_2O. The aromatic region of the IF3 spectrum in 25 mM Na_2HPO_4, pH 7.5, 100 mM NaCl, 1.0 mM EDTA, and 1.0 mM dithioerythritol (physiological phosphate buffer) (Figure 6) is more complex than the spectrum one would obtain from an equimolar mixture of aromatic amino acids (Wüthrich, 1976). For example, the resonances of Tyr-107 C3 and C5 protons are shifted upfield to 6.7 ppm, indicating the ring current influence of a nearby aromatic residue. The methyl proton resonances of IF3 (Figure 6, inset) are shifted upfield. The upfield shifts in the aromatic and methyl resonances are characteristic features of an extensively folded protein with a high degree of secondary and tertiary structure (Wüthrich, 1976). During the course of this work, Paci et al. (1984) reported the 400- and 500-MHz proton NMR spectra of IF3, which appear to be identical with the spectra in Figure 6. We have adopted their peak assignments, which are supported by pH titrations, chemical modification studies, and decoupling experiments, although assignment of tyrosine residues is still tentative.

The aromatic region of the cloacin fragment spectrum in 5.0 mM sodium cacodylate, pH 5.5, and 95 mM NaCl appears

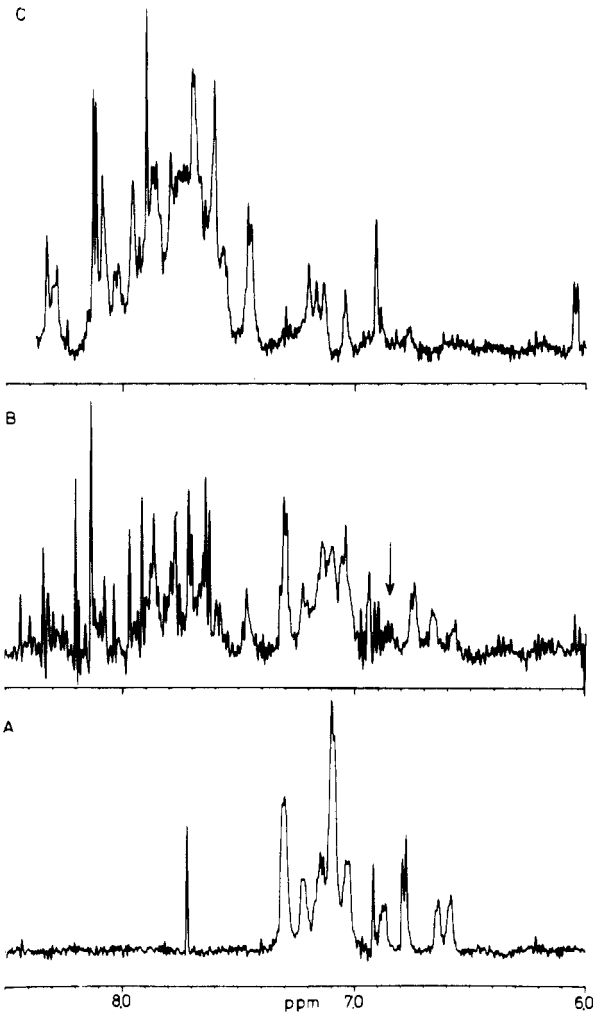

FIGURE 7: 500-MHz proton spectra in ^2H_2O of (A) 0.2 mM IF3, as in Figure 6, (B) 0.12 mM IF3 + 0.12 mM cloacin fragment in 2.0 mM sodium cacodylate, 15 mM Na_2HPO_4, pH 7.3, 98 mM NaCl, 0.6 mM EDTA, and 0.6 mM dithioerythritol, prepared by mixing equimolar amounts of solutions in (A) and (C) (2000 scans), and (C) 0.3 mM cloacin fragment in 5.0 mM sodium cacodylate, pH 5.5, and 95 mM NaCl (5580 scans). The arrow in (B) indicates the location of position of missing Tyr-107 resonances.

in Figure 7C. There is significant overlap between cloacin fragment peaks and IF3 peaks, repeated for comparison in Figure 7A. The samples from spectra A and C of Figure 7 were mixed in the appropriate proportions to yield an equimolar solution of cloacin fragment and IF3, 0.12 mM each in 2.0 mM sodium cacodylate, 15 mM Na_2HPO_4, pH 7.3, 98 mM NaCl, 0.6 mM EDTA, and 0.6 mM dithioerythritol. At these concentrations, 98% of the RNA and protein molecules would be complexed with each other, assuming the IF3/cloacin fragment association constant of 1.8×10^7 M^{-1} calculated above for the CD titration in physiological Tris buffer. This is an approximation, since the buffer mixture in this experiment is similar to physiological Tris buffer in ionic strength and pH but is buffered by phosphate instead of Tris, has sodium for its major cation instead of ammonium, and lacks Mg^{2+}.

The aromatic region of the IF3/cloacin fragment spectrum (Figure 7B) does not show much broadening of either protein or RNA resonances. This indicates high mobility of the residues, which could be the result of a short lifetime for the complex. Neither the Phe nor Tyr-75 resonances seem to be significantly altered by complexing with the cloacin fragment. However, one of the doublets of Tyr-107 has disappeared, either by shifting under the Phe resonances or by broadening beyond recognition.

NMR Spectra in ^1H_2O. The low-field imino proton region of the cloacin fragment spectrum in 1.0 mM sodium cacodylate, 25 mM Na_2HPO_4, pH 7.0, 61.5 mM NaCl, and 1.0 mM EDTA (Figure 8A) shows nine resonances of water-exchangeable hydrogen-bonded protons, which we previously assigned by nuclear Overhauser effects (Heus et al., 1983b) to eight of the base pairs, A15-U34 through G22-C27, in Figure 1A. In the imino proton spectrum of the IF3/cloacin fragment complex in 5.0 mM sodium cacodylate, pH 7.0, and 95 mM NaCl, (Figure 8B), most of the cloacin fragment resonances are still present, implying that the helix is still intact. All of the resonances show some broadening, which is probably due both to the greater molecular weight of the IF3/cloacin fragment complex, more than twice that of the cloacin fragment, and to enhanced imino proton exchange. However, the A20-U29 resonance has almost disappeared from the spectrum, while the peaks belonging to the U19-G30 protons are substantially broadened. These effects were also observed with the cloacin fragment alone, at higher temperatures (Heus et al., 1983b). Furthermore, the upfield shift of the G22-C27 resonance beneath the G18-C31 resonance, which was previously found to occur upon raising the temperature (Heus et al., 1983b), is also found upon complex formation.

When the NaCl concentration of the IF3/cloacin fragment mixture was raised to 1 M (Figure 8C), the broadening of the U19-G30 and A20-U29 resonances was not reversed, as one would expect if IF3 binding to the cloacin fragment were primarily ionic and nonspecific, as it is at low ionic strength.

Discussion

CD Spectra. The CD spectrum of the cloacin fragment RNA in physiological Tris buffer is appropriate for a G-C-rich oligonucleotide in which only about half of the residues are base-paired. The shapes of the CD spectra of the cloacin fragment in both buffers were virtually the same, but the spectra in physiological Tris buffer had a lower peak magnitude than the spectra in the low-salt phosphate buffer. Physiological Tris buffer is the standard assay buffer for ribosome and initiation factor activity (Hershey et al., 1977), except that the $[Mg^{2+}]$ is 1 mM rather than 5 mM. The low-salt phosphate buffer is the same buffer used before in measurements of IF3 binding to homopolynucleotides by filter binding (Wickstrom et al., 1980) and CD titration (Schleich et al., 1980; Wickstrom, 1981).

The magnitudes of the peak and trough in physiological Tris buffer are typical for RNAs in similar solutions. The magnitudes in low-salt phosphate buffer are greater than in physiological Tris buffer. The phenomenon of greater CD peak magnitude in low salt in the absence of Mg^{2+}, than in physiological salt with Mg^{2+} present, as described above for the cloacin fragment, has been seen previously for synthetic polynucleotides (Schleich et al., 1980). It is unlikely that the bases of the cloacin fragment in physiological Tris buffer are less stacked than in low-salt phosphate buffer, but the base–ribose geometry may differ slightly in the two buffers, resulting in different magnitudes.

The titration experiments imply that IF3 destabilizes the secondary structure of the cloacin fragment upon binding. In physiological Tris buffer, IF3 destabilizes the cloacin fragment by only 19%, roughly equivalent to raising the temperature to 41 °C, and appears to bind to only a single site on the RNA, in agreement with nuclease mapping data under similar conditions, but with excess unlabeled tRNA (Wickstrom, 1983).

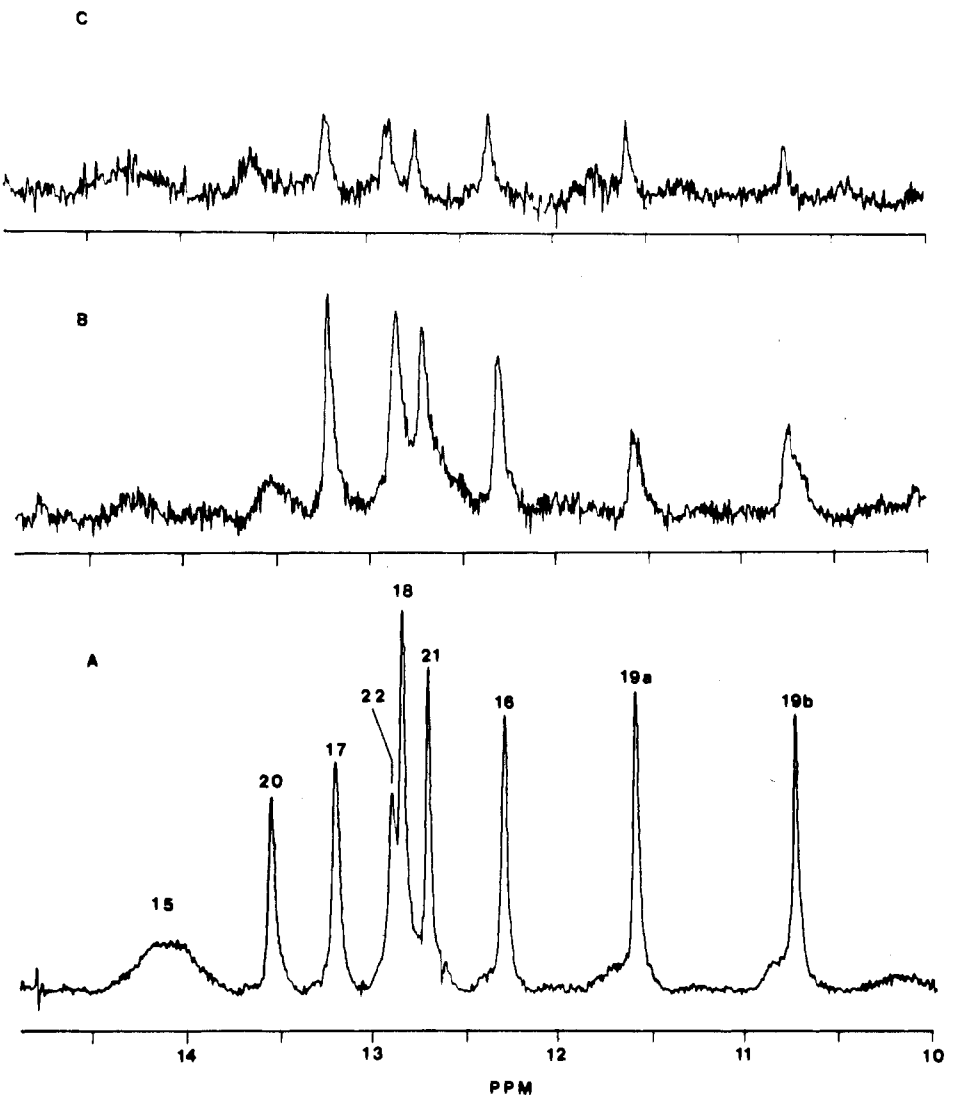

FIGURE 8: 500-MHz proton spectra of (A) 0.7 mM cloacin fragment in 1.0 mM sodium cacodylate, 25 mM Na_2HPO_4, pH 7.0, 61.5 mM NaCl, and 1.0 mM EDTA containing 5% ^2H_2O (4100 scans), with peaks numbered according to base pairs in Figure 1, (B) 0.12 mM IF3 + 0.12 mM cloacin fragment in 5.0 mM sodium cacodylate, pH 7.0, and 95 mM NaCl containing 8% ^2H_2O (3800 scans), and (C) the same as (B) but with 1.0 M NaCl (1500 scans).

The best fit association constant for this titration agrees well with that predicted by the nuclease mapping study, implying a lack of competition with tRNA for binding to IF3, and is close to that for IF3 binding to 30S subunits, 5.8×10^7 M^{-1}, under similar conditions (Weiel & Hershey, 1981).

In low-salt phosphate buffer, IF3 decreases the cloacin fragment peak CD by 35% and appears to bind cooperatively to a pair of sites, unlike the in vivo situation (Wittmann, 1983). The best fit association and cooperativity constants are in excellent agreement with those found for IF3 binding to poly(rA) in the same buffer (Wickstrom, 1981). This result is consistent with the observed lack of sequence specificity by IF3 in low-salt phosphate buffer (Wickstrom, 1974; Wickstrom et al., 1980; Schleich et al., 1980).

NMR Spectra. The NMR spectra carry several implications for complex formation between IF3 and the cloacin fragment. Upon binding, the resonances of Tyr-107, which is involved in IF3 binding to 30S ribosomal subunits (Bruhns & Gualerzi, 1980), shift downfield or broaden a great deal, while the resonances of Tyr-75, which is not essential for IF3 activity (Bruhns & Gualerzi, 1980), are unaffected. These results are in excellent agreement with those of Paci et al. (1985) for IF3 binding to deuterated 30S ribosomal subunits, consistent with the specificity of IF3 binding to the cloacin fragment. Whether or not IF3 undergoes a major conformational change upon binding to the cloacin fragment cannot be determined yet from these one-dimensional spectra.

The imino proton spectra of the IF3/cloacin fragment complex reveal substantial broadening of the U19-G30 peaks, virtual disappearance of the A20-U29 resonance, and an upfield shift of the G22-C27 resonance beneath the G18-C31 resonance. There are two reasonable explanations for these observations. First, some part of the IF3 that comes in close contact with these base pairs might accelerate U19-G30 and A20-U29 imino proton exchange with water and perturb the environment of G22-C27. Second, IF3 binding to the cloacin fragment might destabilize the central and upper parts of the helix. At present, we cannot distinguish between these two possibilities. Proton NMR studies of the binding of *E. coli* $tRNA^{Phe}$ to elongation factor Tu, its natural interaction partner, showed a similar effect on the U-G base pair in the amino acid acceptor stem of $tRNA^{Phe}$ (Hilbers et al., 1983).

The failure of 1 M NaCl to reverse the effects of IF3 on the U19-G30 and A20-U29 resonances or the upfield shift of the G22-C27 resonance may imply that IF3 interaction with the cloacin fragment is largely nonionic and sequence specific at physiological ionic strength and above, which was also found in the nuclease mapping study (Wickstrom, 1983). However,

this interpretation must be considered tentative, since at this stage we lack detailed information on the influence of ionic strength on RNA imino proton resonances in the absence of protein. Additional details of the interaction between the cloacin fragment and IF3 may be obtained by measuring magnetization transfer from side chains in IF3 to nucleotides in the cloacin fragment, an intermolecular nuclear Overhauser effect.

Implications. In the loop of the central hairpin of the cloacin fragment (Figure 1), both adenosines are dimethylated on N6. In methylation-deficient, kasugamycin-resistant mutants, the loop adenosines display less stacking, and the hairpin is more stable as a result (van Charldorp et al., 1981, 1982). Such mutants also require greater concentrations of IF3 for full initiation activity (Poldermans et al., 1979). This observation now may be correlated with destabilization of the hairpin upon IF3 binding, indicated by altered nuclease susceptibility (Wickstrom, 1983), decreased CD, and broadened or shifted NMR peaks. The more stable mutant hairpin is presumably more difficult for IF3 to disrupt, so the association constant of IF3 would be lower than it is for binding to the normal cloacin fragment. As a consequence, a higher IF3 concentration would be required for the same extent of binding. Studies of IF3 binding to the mutant cloacin fragment and mutant 30S ribosomal subunits are under way. Preliminary probes of both the normal and unmethylated mutant cloacin fragment with diethyl pyrocarbonate, in the presence and absence of IF3, suggest that IF3 also sequesters A25 and A26 in the loop of the hairpin (H. A. Heus et al., unpublished results).

It appears from the results reported here and in the nuclease mapping study (Wickstrom, 1983) that IF3 binding to the cloacin fragment is probably its predominant interaction with 30S ribosomal subunits. The existence of some other weak interactions was indicated in sedimentation studies of IF3 binding to 30S subunits lacking the colicin fragment (Laughrea et al., 1978). Our observations suggest that IF3 binding alters secondary structure in the 3′ terminus of 16S rRNA in 30S ribosomal subunits, thus changing the 30S subunit conformation, in accord with some models for IF3 function (van Duin et al., 1976; Pon et al., 1982).

Taking account of the nuclease mapping, CD, NMR, and chemical probe studies of IF3 binding to the cloacin fragment, a detailed model for the interaction may be formulated. IF3 appears to interact specifically with those residues conserved throughout evolution, namely, G1-U13, the base pairs U19-G30 and A20-U29, the hairpin loop residues m^2G23-m_2^6A26, and G36-A38 (van Knippenberg et al., 1984). Many of these residues become more accessible to solvent upon IF3 binding, making some nucleotides, G1, G3, C8, G11, G12, U13, G24, G30, G36, and G37, more susceptible to single-strand-specific nucleases or broadening the imino proton resonances of the conserved base pairs. A few nucleotides, U2, A6, A7, A9, A10, U19, m_2^6A25, and m_2^6A26, are sequestered from nuclease or chemical attack.

The intricacy and specificity of the interaction is indicated by the observation that a given residue may be sequestered by IF3 while its immediate neighbor or base-pair partner is made more accessible. If an A-form structure for the cloacin fragment is assumed, the 13 nucleotides of the 5′ leg are about 3.5 nm long and the hairpin is about 3.0 nm high, adding up to 6.5 nm in the most extended form possible. With a radius of gyration of 1.5 nm and an axial ratio of 3.5 (Gualerzi & Pon, 1981), IF3 is at most 10.5 nm long and 3.0 nm wide. Hence, IF3 is more than large enough to bind to the conserved residues of the cloacin fragment, from the 5′ end to the top of the hairpin, with room left over to bridge the cleft of the 30S ribosomal subunit.

Previous work in our laboratories led us to think that spectroscopic studies might reveal details of changes in the secondary structures of IF3 and the 16S rRNA 3′-terminal cloacin fragment and that such phenomenona might be used to quantitate the strength of IF3 binding to the cloacin fragment. The results presented above verify these expectations and encourage us to pursue the molecular details of this protein–nucleic acid interaction and refine our model by two-dimensional NMR analysis.

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