Cross-linking of *Escherichia coli* Initiation Factor IF-3 to the RNA Moiety of the 30S Ribosomal Subunits†

Cynthia L. Pon, Richard Brimacombe, and Claudio Gualerzi*

ABSTRACT: *Escherichia coli* initiation factor IF-3 labeled in vitro by either reductive alkylation or reaction with *N*-ethylmaleimide can be cross-linked to the 16S rRNA of 30S ribosomal subunits by irradiation with ultraviolet light or reaction with formaldehyde, respectively. In both cases, the cross-linking to the 16S rRNA appears to be direct and not mediated via ribosomal proteins. When cross-linking to 16S and 23S rRNAs is compared at short irradiation times or under conditions which depress nonspecific IF-3 binding, the factor is cross-linked two to three times more efficiently to the 16S than to the 23S rRNA. When cross-linked complexes of 30S and radioactive IF-3 are isolated and subjected to limited hydrolysis with RNase T1, the 30S ribosomes are split into two subparticles which can be electrophoretically resolved; the two particles each contain a characteristic set of proteins with one (band II) containing S4, S5, S6, S8, S15, S18, S20, and S16 or S17, and the 900 nucleotides of the 5′ end and the other (band III) S7, S9, S10, S14, S19 and the remaining 3′ side (minus the last 150 nucleotides) of the 16S rRNA molecule. Cross-linked IF-3 is found unequally distributed between these two ribonucleoprotein particles, even when the irradiation is extrapolated to zero time, with band II containing 80–90% and band III 10–20% of the cross-linked factor. This distribution was found to be identical after both ultraviolet and formaldehyde cross-linking, and with IF-3 labeled by modifying either the lysine residues or the only cysteine residue. The RNA–IF-3 cross-link appears to be labile suggesting that several points of interaction may exist between IF-3 and the 16S rRNA which give rise to several unstable cross-linking bonds.

The topographical localization of the IF-3 binding site on the 30S ribosomal subunit has been attempted by different techniques. The picture emerging from these studies is that, when bound to the ribosome, IF-3 is localized in a region probably adjacent to the subunit interface (Gualerzi et al., 1973) neighboring, at least in part, the 3′ end of the 16S rRNA (van Duin et al., 1975) as well as ribosomal proteins S1, S7, S11, S12, S13, S14, S18, S19, S21 (Hawley et al., 1974; Lake and Kahan, 1975; van Duin et al., 1975; Heimark et al., 1976). Although IF-3 seems to bind to the ribosomes via the 16S rRNA (Gualerzi and Pon, 1973; Pon and Gualerzi, 1976), the cross-linking experiments have so far been more successful in identifying the protein neighbors of IF-3 rather than the region(s) of the RNA adjacent to the bound factor. This situation reflects both the technological gap in the development of protein–protein vs. protein–RNA cross-linking procedures, as well as the greater difficulty inherent in the unequivocal identification of short stretches of a long polynucleotide, such as the 16S rRNA, as compared with the identification of ribosomal proteins, which can easily be achieved by electrophoretic and immunological techniques. As far as IF-3 is concerned, a cross-linking between the factor and the 16S rRNA has so far been obtained by a photosensitization procedure (Cooperman et al., 1977) and by use of 30S ribosomal subunits containing bromouracil (Gualerzi and Pongs, unpublished observations). In neither case, however, has the RNA region engaged in the cross-linking been identified. Only in the cross-linking reaction following periodate oxidation of the RNA has the 3′ end of the 16S been implicated in cross-linking with IF-3 (van Duin et al., 1975). This is a special case, however, since the periodate oxidation exclusively promotes the reactivity of the 3′ end of the RNA molecule. In addition, under similar conditions, the 3′ end of the 16S rRNA can also react with ribosomal protein S1 (Kenner, 1973), as well as initiation factors IF-1 and IF-2 (S. Langberg and J. W. B. Hershey, personal communication), so that all these cross-links may well be the result of the high reactivity of the modified 3′ end and of the ready accessibility of this region of the 16S rRNA in the 30S subunit (Bowman et al., 1971; Santer and Santer, 1973; Noller, 1974).

Recent papers have shown the possibility of cross-linking ribosomal proteins to the rRNA by means of either UV[1] irradiation (Gorelic, 1975; Möller and Brimacombe, 1975) or formaldehyde treatment (Möller et al., 1977). Although the chemistry of these reactions is, to a large extent, unknown, it seems likely that they are not based on group specific reactions. This, in turn, may prove to be advantageous, especially in the case of a multimolecular structure like the ribosome, since, if the reaction does not depend strictly upon the availability of a specific reactive group(s), one is likely to obtain data which reflect more faithfully the points of contact or neighborhoods between proteins and RNA.

In the present study, UV irradiation and formaldehyde treatment as means of promoting cross-links between RNA and protein have been applied to the study of the interactions of initiation factor IF-3 with the 30S ribosomal subunit and the results are presented in this paper.

Materials and Methods

Materials. [^{14}C]Formaldehyde (50 mCi/mmol) and [^{3}H]-*N*-ethylmaleimide (150 mCi/mmol) were obtained from New England Nuclear Corp. Sodium boro[^{3}H]hydride (10.0 Ci/mmol) was obtained from Amersham.

Preparation of Ribosomes, Ribosomal Proteins, and Factors. Ribosomal subunits were prepared from 1 M NH_4Cl

† From the Max-Planck-Institut für Molekulare Genetik, Abt. Wittmann, Berlin-Dahlem, West Germany. *Received July 12, 1977.*

[1] Abbreviations used: UV, ultraviolet; EDTA, ethylenediaminetetraacetic acid; ^{3}H-IF-3, IF-3 labeled with [^{3}H]-*N*-ethylmaleimide; NEM, *N*-ethylmaleimide.

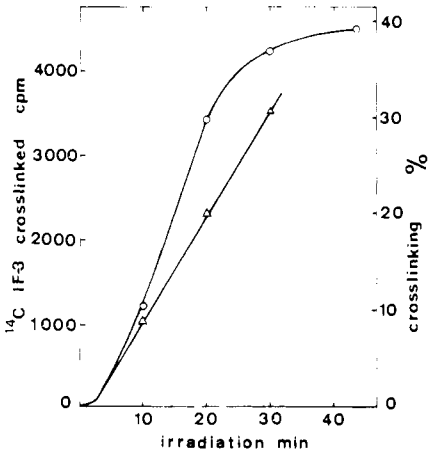

FIGURE 1: Time course of UV-induced cross-linking of ^{14}C-IF-3 to the ribosomal subunits and 16S rRNA. Irradiation and analysis of the cross-linked product were carried out as described in Materials and Methods. Cross-linking to the 30S ribosomal subunits (○) and to 16S rRNA (△).

washed 70S ribosomes of *Escherichia coli* MRE600 and initiation factor IF-3 was purified to electrophoretic homogeneity from the 1 M NH_4Cl ribosomal wash as previously described (Gualerzi et al., 1971; Risuleo et al., 1976). Purification of ribosomal proteins S8 and S19 and reconstitution of the 30S ribosomal subunit were performed essentially as described by Held et al. (1973). Reductive alkylation of IF-3 and ribosomal proteins S8 and S19 was as described (Gualerzi et al., 1973). In vitro labeling of IF-3 with [^{3}H]-*N*-ethylmaleimide will be described in detail elsewhere.

Cross-linking by UV Irradiation. For a typical UV cross-linking reaction the 30S ribosomal subunits in buffer containing 10 mM Tris-HCl, pH 7.7, 10 mM magnesium acetate, and 100 mM NH_4Cl were incubated for 10 min at 37 °C with a two- to fourfold molar excess of purified in vitro labeled IF-3. Aliquots (0.4 mL) of the reaction mixture containing 4 A_{260} units of 30S subunits were then placed in sterilized plastic wells (1.5 cm diameter), which were aligned on a metal plate lying on ice and irradiated with an original hanau "Sterisol" germicidal lamp at a distance of approximately 5 cm. The irradiated samples were then divided into three portions which were treated in the following ways: (a) loaded on sucrose gradients to determine total amount of radioactive IF-3 bound to 30S ribosomes (Pon and Gualerzi, 1976); (b) made 400 mM with respect to NH_4Cl concentration and centrifuged as in a but in gradients containing 400 mM NH_4Cl, to determine the amount of IF-3 cross-linked to the 30S ribosomal subunits; and (c) brought to 100 mM NaCl, 1 mM EDTA, 1% sodium dodecyl sulfate in 10 mM sodium maleate buffer (pH 6.8) and incubated for 10 min at 37 °C, and then centrifuged at 48 K rpm in a Spinco SW 60 Ti rotor for 5 h at 13 °C on a 10–30% sucrose gradient containing 100 mM NaCl, 1 mM EDTA, and 10 mM sodium maleate buffer (pH 6.8) to determine the amount of IF-3 cross-linked to the 16S rRNA.

RNase T1 Digestion. The 30S–IF-3 cross-linked complex to be used in the T1 hydrolysis experiment was isolated on sucrose gradients containing 400 mM NH_4Cl as in b above. The fractions containing the complex were pooled and the ribosomes were precipitated by addition of 0.7 volume of cold ethanol. The pellets obtained by centrifugation were then dissolved in 20 mM Tris-HCl, pH 7.8, 60 mM NH_4Cl, and 5 mM magnesium acetate to 20–25 A_{260} units/mL. The samples were then made 2 M in urea and hydrolyzed with RNase T1 (17 enzyme units/A_{260} unit of 30S subunit) for 1.5 h at 24 °C as previously described (Rinke et al., 1976).

Electrophoretic Procedures. The hydrolysis products were separated by electrophoresis on 5% polyacrylamide gel slabs at pH 6.0 as described (Rinke et al., 1976) using 2-cm wide slots. After electrophoresis, a 1.3 cm × 10 cm strip was cut from the center of each gel slot and the remainder of the gel was stained with methylene blue (Dahlberg et al., 1969). The 1.3-cm strip was further cut into a 0.5 × 10 cm and a 0.8 × 10 cm strip both of which were simultaneously sliced into 1.7-mm fractions. The 5-mm wide fractions were analyzed for radioactivity while the 8-mm wide slices were kept for application as appropriate to a 5% polyacrylamide gel containing sodium dodecyl sulfate for analysis of the RNA as previously described (Rinke et al., 1976). This second gel was both stained for RNA and analyzed for IF-3 radioactivity as above.

Electrophoretic analysis of proteins was performed in 12.5% polyacrylamide–sodium dodecyl sulfate gel slabs (Laemmli, 1970; Studier, 1973). Autoradiograms of slab gels were obtained using Agfa Curix RP1 x-ray films which were scanned with an Ortec Model 4310 densitometer.

Cross-linking by Formaldehyde Treatment. Complexes of 30S subunit and ^{3}H-IF-3 (IF-3 labeled with [^{3}H]-*N*-ethylmaleimide) were prepared as above. Unbound IF-3 was removed from 30S subunit by standard sucrose gradient centrifugation. The fractions containing the 30S–IF-3 complex were pooled and the complex was ethanol precipitated as described above. The pellets were then resuspended in 50 mM triethanolamine hydrochloride, pH 7.8, 50 mM KCl, 5 mM magnesium acetate to a concentration of 40 A_{260} units/mL and incubated for 45 min at 37 °C with either 0%, 0.03%, or 0.05% formaldehyde (Möller et al., 1977). Following the reaction the samples were made 2 M in urea and hydrolyzed with RNase T1 as described above. The hydrolyzed samples were separated first on the pH 6.0 gel and subsequently analyzed on a 5% gel containing sodium dodecyl sulfate as described above with the exception that both gel and reservoir buffers contained 0.03% formaldehyde and that triethanolamine was used in place of Tris (Möller et al., 1977). The entire experiment from formaldehyde incubation to final RNA analysis was carried out as quickly as possible (within 15 h) in view of the reversibility of the formaldehyde reaction (Möller et al., 1977).

Results

The binding of IF-3 to the 30S ribosomal subunit is extremely sensitive to high salt concentrations (Pon and Gualerzi, 1976). However, when the 30S ribosomal subunits are incubated with IF-3 and then subjected to UV irradiation for different lengths of time, increasing amounts of IF-3 can be recovered in association with the 30S ribosomes after centrifugation in sucrose gradients containing high salt concentrations. Nonirradiated controls show no radioactive IF-3 associated with 30S ribosomes in a high salt containing sucrose gradient (Figure 1). It can be seen in the same figure that the amount of IF-3 bound to 30S under high salt conditions steadily increases until it reaches a plateau after about 30 min, at a level which represents over 35% of the IF-3 originally bound to the 30S subunits. Longer irradiation times resulted in increasing and nonsaturating cross-linking probably due to extensive unfolding of the 30S ribosome (not shown). After treatment of the ribosomes with sodium dodecyl sulfate and isolation on a sucrose gradient, nearly all this high salt resistant radioactive IF-3 cross-linked to the 30S ribosomes can be recovered in association with the 16S rRNA. The small amount of radioactivity which was high salt resistant but not recovered in as-

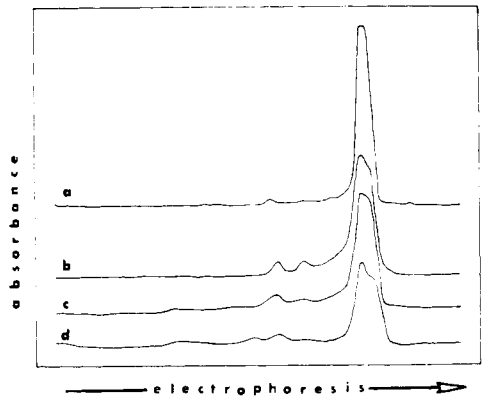

FIGURE 2: Lack of UV-induced protein-protein cross-link involving IF-3 and ribosomal proteins. Each reaction mixture (0.4 mL) contained 22.5 μg of ^{14}C-labeled IF-3 (a and d) and either 4.0 A_{260} units of 30S ribosomal subunits (b), or 3.35 A_{260} units of 16S rRNA (c). The samples were incubated for 10 min at 37 °C and then irradiated (with the exception of sample a) for 30 min as described in Materials and Methods. After irradiation, samples b and c were treated with sodium dodecyl sulfate and centrifuged as described in Materials and Methods (procedure C). The fractions containing the 16S rRNA and cross-linked ^{14}C-IF-3 were pooled. These samples as well as sample c were then incubated for 2 h at 37 °C with 5 μg of pancreatic RNase. Bovine serum albumin carrier (50 μg), sodium deoxycholate (100 μg), and trichloroacetic acid (ca. 10%) were added. After 20 min at 0 °C, the samples were centrifuged and the pellets dissolved in a small volume of electrophoresis buffer and electrophoresed. The gels were dried and autoradiographed, and the scans of the autoradiograms are presented above.

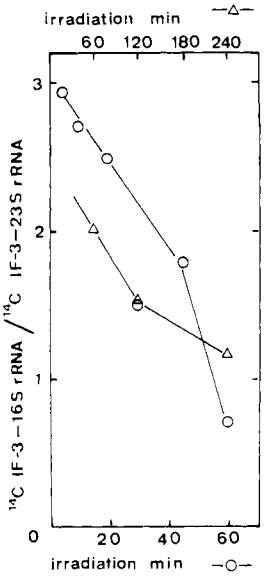

FIGURE 3: Preferential cross-linking of ^{14}C-IF-3 to the 16S rRNA. In the first experiment (○), the irradiations were carried out as described in Materials and Methods with the exception that each sample contained 10 μg of ^{14}C-IF-3, and a mixture of 30S ribosomal subunits (4.0 A_{260} units) and 50S ribosomal subunits (8.0 A_{260} units) and that the Mg^{2+} concentration was lowered to 5 mM. The amount of IF-3 cross-linked to the 23S and 16S rRNA was determined following sodium dodecyl sulfate treatment and sucrose gradient centrifugation as described in Materials and Methods. In the second experiment (△), the UV irradiation was carried out essentially as described in Materials and Methods, but each sample (0.4 mL) contained 20 μg of ^{14}C-IF-3, 20 mM magnesium acetate, 200 mM NH_4Cl, and 500 μg of *E. coli* MRE 600 tRNA (Boehringer, Mannheim) and either 5.0 A_{260} units of 50S ribosomes or 2.5 A_{260} units of 30S ribosomes. The samples were irradiated for the indicated times (longer times of irradiation were necessary due to the high absorbance of the samples) and the amount of IF-3 cross-linked to the 23S or 16S rRNA was determined as described in Materials and Methods.

sociation with the 16S rRNA could not be accounted for by any stable protein-protein cross-link which could be detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (not shown).

The finding of an association between IF-3 and the 16S rRNA suggests but does not prove per se a direct cross-linking between the factor and the RNA since the association could be mediated via one or more ribosomal proteins. To rule out this possibility, radioactive IF-3 was UV-irradiated alone, or after binding to either 30S ribosomal subunits or deproteinized 16S rRNA. After treatment with sodium dodecyl sulfate the 16S rRNA-^{14}C-IF-3 cross-linked complexes were isolated on sucrose gradients and treated with pancreatic RNase. The samples were then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to detect any radioactivity of higher molecular weight than IF-3, which could have arisen from a cross-linked product between IF-3 and an unknown 30S ribosomal protein. Figure 2 presents a densitometer tracing of an autoradiography of such a gel. As seen in the figure, although some degradation of IF-3 occurs in all irradiated samples (as indicated by the light shoulder), in all cases the bulk of the radioactivity migrates to the same position as an unirradiated IF-3 marker, and no differences are seen between the IF-3 samples irradiated alone, in the presence of deproteinized 16S rRNA, or after binding to the 30S ribosomal subunits. These results indicate that the cross-linking between IF-3 and the 16S rRNA is direct rather than mediated by a ribosomal protein.

When the 50S ribosomal subunits were UV-irradiated with radioactive IF-3 in an experiment similar to that of Figure 1, increasing amounts of 23S-IF-3 cross-links were found. In this case, however, no saturation was reached and the amount of IF-3 cross-linked to the 23S rRNA eventually exceeded one IF-3 per 23S molecule (not shown). This finding is reflected by the results presented in Figure 3, where the amounts of IF-3 cross-linked to the 16S and 23S rRNAs were compared as a function of the time of UV irradiation of radioactive IF-3 and a mixture of 30S and 50S ribosomal subunits. It can be seen from the figure that the ratio of the radioactivity cross-linked to the 16S rRNA to that found in the 23S rRNA varies from about three for the shortest irradiation period to less than one for the longest time. An analogous result was obtained when the 50S and the 30S ribosomal subunits were individually irradiated with radioactive IF-3 but under conditions (presence of tRNA, high Mg^{2+} and NH_4^+ concentrations) which are expected to depress nonspecific IF-3 binding (Figure 3). A partial explanation for these findings is provided by the experiment shown in Figure 4 in which 30S or 50S ribosomal subunits were UV irradiated as usual, but in the absence of IF-3, and their IF-3 binding capacity was then determined by sucrose gradient centrifugation (Pon and Gualerzi, 1976). As seen in the figure, while the irradiation of the 30S ribosomal subunits resulted in the progressive inactivation of the IF-3 binding site, the analogous irradiation of the 50S ribosomal subunits promoted substantial binding of the factor to these particles. This is probably due to the unfolding of the 50S ribosomal subunits as previously suggested (Newton et al., 1975).

When a 30S-^{14}C-IF-3 cross-linked complex isolated from sucrose gradients containing a high salt concentration is subjected to limited T1-RNase hydrolysis, three ribonucleoprotein bands (I, II, III) can be detected by polyacrylamide gel electrophoresis (Figure 5). These three bands correspond to those which have previously been well characterized (Yuki and Brimacombe, 1975) with band I consisting of more or less intact 30S ribosomes, band II containing 900 nucleotides of the 5′ end, and band III the remaining 3′ end of the 16S rRNA

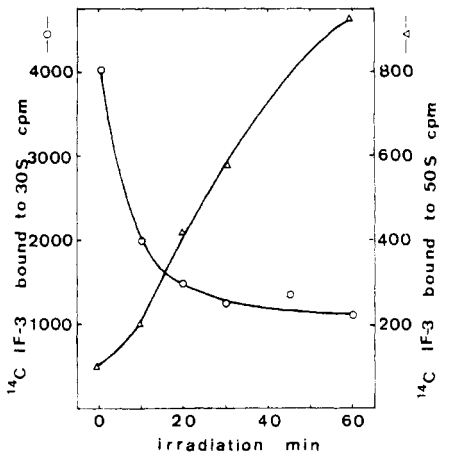

FIGURE 4: Effect of UV irradiation on the IF-3 binding capacity of 30S and 50S ribosomal subunits. Both 50S and 30S subunits were irradiated for the indicated times under conditions identical with those described in Materials and Methods with the exception that IF-3 was not present during the irradiation period. The IF-3 binding capacity of the treated particle was determined as described (Pon and Gualerzi, 1976) using 0.5 A_{260} unit of 30S (○) or 1.0 A_{260} unit of 50S subunits (△).

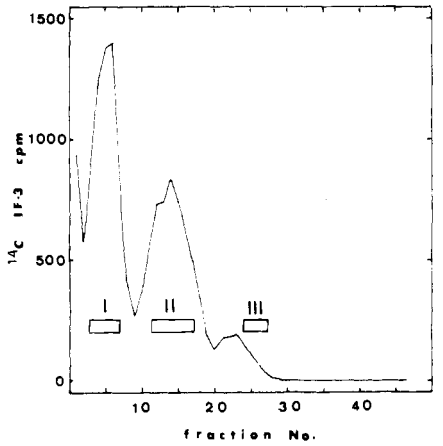

FIGURE 5: RNase T1 hydrolysis profile of the UV-induced cross-linked complex between 30S subunits and ^{14}C-IF-3. The cross-linked complex was formed, purified in high salt containing sucrose gradients, hydrolyzed with RNase T1, and electrophoresed as described in Materials and Methods. The darkened areas correspond to the position of the methylene blue stained bands I, II, and III.

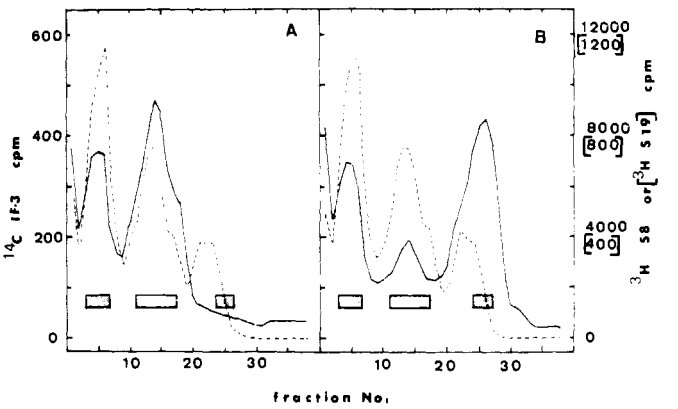

FIGURE 6: RNase T1 hydrolysis profile of the UV-induced cross-linked complex between 30S subunits containing ^{3}H-S19 or ^{3}H-S8 and ^{14}C-IF-3. The experiment was analogous to that of Figure 5 with the exception that reconstituted 30S ribosomes containing either ^{3}H-S8(A) or ^{3}H-S19(B) were used. Preparation of the labeled ribosomal proteins and reconstitution of the 30S subunits were performed as described in Materials and Methods. ^{14}C-IF-3 (- - -); ^{3}H-S8 or ^{3}H-S19 (—).

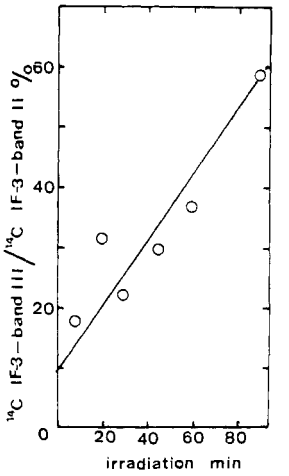

FIGURE 7: Distribution of ^{14}C-IF-3 between band II and band III as a function of time of UV irradiation. The experiment was carried out essentially as that of Figure 5 with the exception that the time of UV irradiation was varied as indicated.

with the exception of the last 150 nucleotides. In addition, bands II and III are characterized by well-defined sets of proteins (Morgan and Brimacombe, 1973).

Examination of the IF-3 radioactivity profile shows that aside from the radioactive IF-3 still associated with band I (the whole 30S particle), the rest of the radioactivity is distributed between two peaks: the first coincides with the stain in band II and the second minor peak has a mobility intermediate between the stains of band II and band III. The lack of coincidence between the minor radioactive peak and the stained bands could arise from either further cleavage of band II or a retarding of band III due to the presence of the factor. To solve this problem the UV-cross-linking experiment and the subsequent partial digestion with T1 ribonuclease were repeated using reconstituted 30S ribosomes containing either ^{3}H-labeled S8 or S19. S8 is known to be present exclusively in Band II while S19 is found predominantly in band III (Morgan and Brimacombe, 1973). The results of Figure 6A clearly show that the band II stain coincides with both ^{14}C-IF-3 and ^{3}H-S8 radioactivities. On the other hand, while the main ^{3}H-S19 peak coincides with the stained band III, a pronounced shoulder of the S19 radioactivity coincides with the minor IF-3 radioactivity peak migrating to a point between stained bands II and III (Figure 6B). Such an S19 shoulder was not observed in control samples irradiated in the absence of IF-3 (not shown). This finding, together with the absence of S8 radioactivity in this region, indicates that the slower moving IF-3 peak is indeed related to band III, which is retarded by the presence of the factor and not to a further degradation product of band II. The amount of radioactive IF-3 associated with band III varied to some extent from experiment to experiment but was approximately 20% of that in band II for a typical 30-min irradiation experiment. Since the radioactivity ratio between band II and band III tended to decrease with increasing doses of irradiation, it was important to determine whether the association of IF-3 with band III was an artifact of ribosome unfolding. To establish this point, radioactive IF-3 was first bound to 30S ribosomal subunits and then subjected to UV irradiation for different lengths of time. The ratio of IF-3 radioactivity between band II and band III was determined for each time of irradiation and the results are plotted in Figure 7. It can be seen from the figure that upon extrapolation to zero time there was still a small but significant proportion (10–15%) of radioactivity in the band III region.

Further analysis aimed at identifying more precisely the regions of the RNA cross-linked to the IF-3 molecule were carried out by transferring bands II and III to sodium dodecyl sulfate–polyacrylamide gels (see Materials and Methods) but, unfortunately, an association between IF-3 and RNA fragments could no longer be detected, indicating that the cross-link is unstable (see Discussion).

In another series of experiments the cross-linking of IF-3 to the 30S ribosomes was attempted by means of formaldehyde treatment (Möller et al., 1977). For this reaction IF-3 was labeled with [^{3}H]NEM instead of by reductive alkylation with formaldehyde. This reaction with NEM results only in the modification of the single -SH residue of IF-3 yielding a labeled protein of reasonably high specific activity with a negligible decrease in activity as judged by three different criteria (Gualerzi and Pon, in preparation).

Preliminary experiments showed that formaldehyde at concentrations between 0.03 and 0.05% caused a substantial cross-linking of IF-3 to the RNA as evidenced by the comigration of the 16S rRNA with ^{3}H-IF-3 on a sodium dodecyl sulfate containing gel, while in controls minus formaldehyde no trace of radioactive IF-3 was found in association with the RNA. When RNase T1 hydrolysis experiments similar to those of Figures 5 and 6 were carried out with 30S–^{3}H-IF-3 formaldehyde cross-linked complexes, a profile (Figure 8A) very similar to that obtained after UV irradiation (cf. Figure 5) was found. Also in this case, the ratio between IF-3 radioactivity in bands II and III is about 4:1 and band III is again displaced by IF-3. The proportion of IF-3 radioactivity remaining with band I, however, was larger after formaldehyde cross-linking than after UV cross-linking. Since this was a constant finding one may argue that upon formaldehyde cross-linking the presence of IF-3 somehow hinders the action of RNase T1 and/or the splitting of the 30S subunit.

Analysis of the RNA–IF-3 complex in a sodium dodecyl sulfate gel system (see Materials and Methods) showed that, in contrast to the UV case, a clear cross-linking of the factor to the RNA has occurred as evidenced by the comigration of the RNA stain with the ^{3}H radioactivity (Figure 8B and C). Peaks of ^{3}H-IF-3 linked to the RNA can be seen in the RNA derived from band I and band II but no peak corresponding to band III could be detected (Figure 8D). A weak cross-linking also in this region cannot be excluded, however, in view of the generally low level of radioactivity. The very low yields of other proteins which could be recovered cross-linked by formaldehyde to band II under these conditions (Möller et al., 1977) make it inconceivable that the observed association of IF-3 with the rRNA could be due to a cross-link of the type IF-3–ribosomal protein–rRNA.

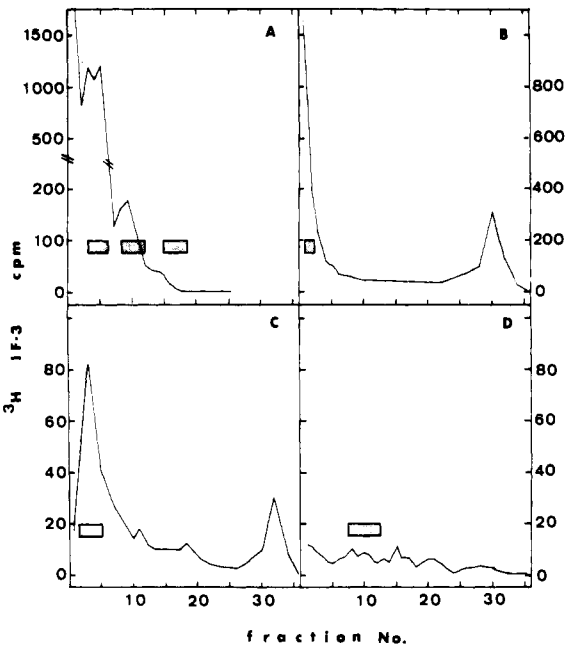

FIGURE 8: RNase T1 hydrolysis profile of formaldehyde-induced cross-linked complex between 30S subunits (A) or rRNA fragments (B–D) and ^{3}H-IF-3. The formation of the formaldehyde-induced cross-link between 30S subunits and ^{3}H-IF-3 was carried out as described in Materials and Methods. The samples were then subjected to limited RNase T1 hydrolysis and electrophoretic analysis as described in Materials and Methods (A). Slices corresponding to band I, band II or to both stain and radioactivity of band III were pooled and the rRNA–^{3}H-IF-3 cross-linked complex therein was analyzed by a second electrophoresis in the presence of sodium dodecyl sulfate (B, C, and D, respectively; cf. Materials and Methods).

Discussion

The results of this paper show that initiation factor IF-3, labeled in vitro by reductive alkylation, can be cross-linked to the 30S ribosomal subunit by UV irradiation. This reaction is dose dependent and after 30 min of irradiation a plateau is reached at a level where more than 35% of the IF-3 bound to the ribosomes becomes cross-linked to the 30S subunits as judged by sedimentation through sucrose gradients containing a high salt concentration. This cross-linking is primarily, if not entirely, due to a direct cross-linking between the 16S rRNA and the IF-3; no protein–protein cross-linking was detected under the same conditions of irradiation.

In addition to cross-linking to the 30S subunits, IF-3 has been cross-linked to ribosomal proteins L2 and L19 of the 50S subunit with dimethyl suberimidate (Sobura et al., 1977) and to the 3′ end of the 23S rRNA following periodate oxidation (van Duin et al., 1975).

When the UV irradiation is carried out with 50S ribosomal subunits, cross-linking between IF-3 and 23S rRNA can also be demonstrated. The cross-linking to the 50S subunits does not reach a plateau, however, indicating that this cross-linking may be due to the progressive unfolding of the 50S structure. Such a loss of specificity upon unfolding of the large subunit has previously been observed (Newton et al., 1975) and seems to be confirmed by the present finding that, while the irradiation of the 30S subunits alone progressively inactivates the IF-3 binding site, the irradiation of 50S alone produces artificial binding sites. Whatever the nature of the interaction (physiological or artifactual) between IF-3 and 50S ribosomal subunits evidenced by the UV-induced irradiation, it should be noted that the direct comparison between the IF-3 cross-linked to the 16S vs. 23S rRNA showed two to three times more cross-linking (on a molar basis) to the 16S RNA whenever very short periods of irradiation ($\leq$10 min) or conditions depressing nonspecific binding were chosen.

When the IF-3–30S cross-linked complexes produced by UV irradiation are isolated, subjected to limited digestion with RNase T1 followed by electrophoresis, the bulk of the IF-3 radioactivity (80–90%) is found in association with a ribonucleoprotein particle (band II) which contains the first 900 nucleotides from the 5′ end of the 16S rRNA and the ribosomal proteins S4, S5, S6, S8, S15, S18, S20, and either S16 or S17 (Morgan and Brimacombe, 1973; Rinke et al., 1977). A minor portion of the IF-3 radioactivity (10–20%) is found in association with another ribonucleoprotein particle (band III) which contains an RNA stretch extending from the O′ fragment to approximately 150 nucleotides before the 3′ end of the 16S rRNA and the ribosomal proteins S7, S9, S10, S14, and S19

(Yuki and Brimacombe, 1975). Although the amount of IF-3 cross-linked to the latter ribonucleoprotein particle tends to increase with time of irradiation, extrapolation of the irradiation to zero time shows that the association of IF-3 with this region of the ribosome is real and not due to the creation of additional binding sites due to the unfolding of the 30S subunit.

IF-3 labeled in vitro by modification of its only sulfhydryl group was also cross-linked to the 30S ribosomal subunit via the 16S rRNA by means of formaldehyde reaction. Upon T1 digestion the distribution of the IF-3 radioactivity between band II and band III was identical with that found following UV irradiation in spite of the fact that the two cross-linking processes are different and IF-3 was modified in different groups. This is a strong indication that the overall localization of IF-3 on the 30S particle emerging from these studies is likely to correspond to the physiological one.

In the past few years, some evidence has accumulated to indicate that the process of initiation, in general, and mRNA recognition, in particular, involve the 3′ end of the 16S rRNA as well as a number of ribosomal proteins (among them S1) believed to be associated with this region of the RNA molecule (Steitz, 1977). It should be noted, however, that neither the colicin E3 cleavage of the 16S rRNA (Thibault et al., 1972) nor the removal of the colicin fragment seems to abolish the binding of IF-3 (Wahba, personal communication) and that the results of this paper, although showing some interaction between IF-3 and the 3′-end side of the 16S rRNA, indicate that the main interaction takes place at the 5′-end side of the molecule.

It has previously been reported that formaldehyde RNA-protein cross-links are easily and spontaneously reversible (Möller et al., 1977), so it is not surprising that this is found also in the case of IF-3. A different picture is obtained, however, following UV-induced cross-linking of the 30S ribosomal subunits. In this case, only one protein (S7) has been found cross-linked to the 16S rRNA and the cross-linked product appears to be particularly stable (Möller and Brimacombe, 1975). The finding that the UV-induced cross-linking between IF-3 and 16S rRNA is labile speaks against an analogy between S7 andIF-3 as far as the nature of the cross-linking bonds is concerned. It is also possible that several separate weak points of interaction may hold together the 16S rRNA and the factor. In favor of this hypothesis is the fact that the time course of IF-3 cross-linking to both 30S and 16S rRNA shows a lag of about 2–3 min (cf. Figure 1). This time may be necessary for the formation of sufficient points of interaction before a cross-linked product can be detected.

The unusual behavior of IF-3 in gel filtration (Gualerzi et al., 1971) and the finding of a large number of ribosomal proteins in cross-linking complexes with it (Hawley et al., 1974; Lake and Kahan, 1975; van Duin et al., 1975; Heimark et al., 1976) suggest that the factor may be an elongated protein. The results of the present cross-linking experiments are compatible with the fact that either IF-3 is an elongated protein which reaches "distant" regions of the 30S ribosomal subunit or that IF-3 is not elongated but binds to a site of the 30S subunit where "distant" regions of the 16S rRNA primary sequence are found in proximity with each other. Experiments are now in progress to determine the actual shape and the dimensions of the IF-3 molecule. In either case, it is attractive to envisage a situation in which the initiation factor spans from one region of the ribosome where it has its main "structural" points of interaction to another region where it engages in its "functional" interaction.

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