Chain initiation factor 3 crosslinks to *E. coli* 30S and 50S ribosomal subunits and alters the UV absorbance spectrum of 70S ribosomes

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ABSTRACT

We report a direct procedure to determine the proteins near the IF-3 binding site in purified 30S and 50S ribosomal subunits. This procedure introduces only limited numbers of cleavable crosslinks between IF-3 and its nearest neighbors. The cleavable crosslinking reagent, 2-iminothiolane, was used to crosslink IF-3 in place to both 30S and 50S subunits. Ribosomal proteins S9/S11, S12, L2, L5 and L17 were found, by this approach, to be in close proximity to the factor in purified IF-3-subunit complexes.

In addition, IF-3 was shown to alter the ultraviolet absorbance spectrum of *E. coli* 70S ribosomes at 10 mM Mg²⁺. The magnitude of the observed difference spectrum at a constant IF-3/ribosome ratio of 1.0, is linearly dependent upon ribosome concentration over the range 5 nM - 55 nM. Titration experiments indicated that the observed effect is maximal at an IF-3/ribosome ratio of approximately 1.0. These results are taken to indicate a conformational change in the 70S ribosome induced by IF-3.

INTRODUCTION

Initiation Factor 3 (IF-3) is intimately involved in the initiation of protein synthesis in *E. coli* (1,2). Despite a decade of intensive study, the precise role of IF-3 in initiation, and the precise mechanism by which it acts, have yet to be unambiguously defined. Indeed, IF-3 has been shown to possess diverse functions, which at first glance appear unrelated to one another. IF-3 may act as a dissociation factor, displacing the equilibrium

towards dissociation when added to ribosome solutions containing 1.0-6.7 mM Mg²⁺ (3,5). However, at high Mg²⁺ concentrations, where IF-3 is not able to promote dissociation, the factor is able to stimulate the translation of synthetic messengers (6,7). In addition, IF-3 seems to be required for the binding of natural messengers during the formation of the 70S initiation complex (4), as well as for the binding of synthetic messengers at 5 mM Mg²⁺ (9). Whether these are truly separate functions, or different aspects of some general IF-3 mechanism remains to be seen.

It was previously shown that IF-3 can be crosslinked to the 30S ribo-

somal protein S12 in purified 30S particles using dimethylsuberimide as the crosslinker (10). The identification of the specific crosslink was demonstrated by dodecyl sulfate gel electrophoresis and by immunological coprecipitation in double diffusion agarose plates. Analysis of nearest neighbors by this procedure is far from ideal, as it could result in ambiguous results because of the possibility of building larger aggregates than dimers and of the drastic measures, such as ammonolysis, that have to be taken to cleave covalent linkers between proteins.

We have developed, and report here, a direct procedure to determine the proteins in the neighborhood of the IF-3 binding site in purified subunits. This method introduces only limited numbers of cleavable crosslinks between IF-3 and its nearest neighbors.

In addition, IF-3 is shown to alter the ultraviolet absorbance spectrum of *E. coli* 70S ribosomes at 10 mM Mg^{2+} . The magnitude of the observed difference spectrum at a constant IF-3/ribosome ratio of 1.0 is linearly dependent upon ribosome concentration over the range 5 nM - 55 nM. Titration experiments indicate that the observed effect is maximal at an IF-3/ribosome ratio of approximately 1.0. These results are taken to indicate a conformational change in the 70S ribosome induced by IF-3.

MATERIALS AND METHODS

Preparation of Ribosomal Subunits

Ribosomes were prepared from freshly grown *E. coli* MRE600 cells and were washed twice in 1.0 M NH_4Cl . Subunits were purified from 70S ribosomes on 10-30% sucrose density gradients containing 10 mM Tris-HCl, pH 7.5, 0.5 mM $MgCl_2$, 100 mM NH_4Cl according to Traub *et al.* (11). Radioactive ribosomal subunits were prepared from *E. coli* MRE600 which had been grown in Tris-salts medium (12) containing 300 μCi [^{14}C]amino acids mixture in 100 ml culture. The specific activity of the ribosomal proteins was 700,000 cpm/mg.

Crosslinking Reagents

2-Iminothiolane (methyl 4-mercaptobutyrimide) was purchased from Pierce Chemical Co. and stored at -20° under N_2 . Only lots which were a white crystalline product were used.

Electrophoresis

Dodecyl sulfate polyacrylamide gel electrophoresis was performed according to Weber and Osborn (13). The two dimensional electrophoresis system of Kaltschmidt and Wittmann (14) was used to separate ribosomal proteins. Radio-

activity was determined by the method of Basch (15) using NCS Tissue Solubilizer (Amersham) to extract the counts.

Antisera

Mouse anti-IF-3 antiserum was prepared as previously described (10). Goat anti-mouse IgG was purchased from Miles-Yeda.

Preparation of IF-3

The factor was purified from the 1.0 M NH_4Cl wash of *E. coli* MRE600 ribosomes as previously described (16). The factor was further purified by passing it over a 2.5 x 150 cm Sephadex G-150 superfine column in 20 mM Tris-Cl, pH 7.5, 500 mM NH_4Cl , 5% glycerol. It was concentrated by ultrafiltration through an Amicon UM-2 membrane.

Crosslinking of IF-3 with 2-Iminothiolane

IF-3, which was dialyzed against a buffer containing 20 mM Bicine (N,N-bis(2-hydroxyethyl)glycine), pH 8.5, 10 mM magnesium acetate, 100 mM KCl and 3% 2-mercaptoethanol, was incubated with 10 mM 2-iminothiolane for 30 min at 0°. Excess reagent was removed by dialysis against buffer A (10 mM Tris-Cl, pH 7.5, 10 mM magnesium acetate, 0.1 M NH_4Cl) containing 3% 2-mercaptoethanol. A mixture of five molar equivalents of modified IF-3 (0.48 mg), 1 equivalent of ^{14}C -labeled 30S ribosomal subunits (4 mg, 775,000 cpm/mg) and 3% 2-mercaptoethanol was incubated for 5 min at 37°. After dialysis against buffer A, the mixture was incubated with 40 mM H_2O_2 for 30 min at 0°. The same reaction was repeated with 50S ribosomal subunits. A mixture of 0.48 mg of modified IF-3, 8 mg (638,000 cpm/mg) of ^{14}C -labeled 50S subunits and 3% 2-mercaptoethanol was first incubated for 5 min at 37°, dialyzed against buffer A and then incubated with H_2O_2 as described above. The reaction mixtures having 30S and 50S subunits were diluted to 10 ml with buffer A containing 1.0 M NH_4Cl and centrifuged for 4 h at 65,000 rpm in the Beckman 65 rotor. After suspension of the pellets in 0.5 ml of buffer A, 2,100,000 cpm (724,137 cpm/mg 30S subunits) and 3,625,000 cpm (627,321 cpm/mg 50S subunits) were recovered in the reaction mixtures. The dissociation of the ribosomal proteins and the immunoprecipitation with anti-IF-3-antiserum is discussed in "Results."

U.V. Difference Spectroscopy

Ultraviolet difference spectra were recorded on a Cary 118 spectrophotometer. Hellma quartz rectangular tandem cells (path length = 0.4375 cm for each chamber) were used for most measurements, with the experimental arrangement shown in Figure 1. Solutions were prepared as follows: A solution of ribosomes in buffer was prepared by adding a small

Fig. 1. Experimental arrangements for UV difference spectroscopy

Solutions were placed into Hellma quartz rectangular tandem cells as follows: (A) buffer + IF-3; (B) reference ribosome solution; (C) buffer; and (D) sample solution. All measurements were made in a Cary 118 spectrophotometer.

volume of concentrated stock ribosomes to the desired volume of buffer containing 10 mM Tris-Cl, pH 7.5, 50 mM KCl and 10 mM MgCl₂ to give a concentration of 1-2 A₂₆₀/ml. Aliquots of 5 ml were then pipetted into separate tubes. IF-3 was added to give the desired molar ratio of IF-3/ribosome (sample solution), and the same volume of buffer was added to a second tube (reference solution). The identical volume of IF-3 that was used in the sample solution was added to 5 ml of buffer (IF-3 solution). Tubes were stoppered, incubated at 37° for 15 minutes, and cooled to room temperature. The difference spectrum was recorded immediately by placing the solutions into the appropriate chambers of the cuvettes as indicated in Figure 1, and rinsing each chamber 3 times with the appropriate solution. The baseline was determined by recording the spectrum of reference solution/buffer versus reference solution/buffer.

Sedimentation Velocity

Sedimentation velocity experiments were performed on a Beckman Model E analytical ultracentrifuge equipped with a U.V. scanner. Solutions identical to those used for spectroscopic measurements were centrifuged at 22,000 rpm, at 20°. S values were determined by using conventional procedures.

RESULTS

The general scheme used to determine which proteins are adjacent to IF-3 in purified subunit-IF-3 complexes is outlined in Figure 2. In step 1, the reagent 2-iminothiolane (17) reacts with lysine amino groups of purified unlabeled IF-3 and any excess reagent is dialyzed away,

Fig. 2. Crosslinking of IF-3 to ribosomal proteins with 2-iminothiolane
This was performed as described in Materials and Methods.

always under reducing conditions (3% 2-mercaptoethanol). In step 2, the amidine derivative of IF-3 containing sulphhydryl groups is allowed to bind to the radioactive ribosomal particles, and the complex is dialyzed to remove most of the 2-mercaptoethanol. It is then oxidized by the addition of H_2O_2 to 40 mM to form a disulfide bond between the amidine derivative of IF-3 and any protein in the immediate proximity which has a free sulphhydryl group in an accessible orientation. Then, in step 3, the proteins are dissociated by the LiCl-urea method (18) and reacted with mouse anti-IF-3-antiserum (step 4). The immunoprecipitation is brought to completion by the addition of goat anti-mouse IgG antiserum in step 5. The immunoprecipitate which is then washed three times in 2.0 ml phosphate buffered saline should therefore contain only the antibodies and IF-3 crosslinked to its radioactive neighbors. In step 6, the complexes are reduced to cleave the crosslink between IF-3 and the radioactive ribosomal proteins, and they are analyzed by two-dimensional gel electrophoresis in the presence of cold ribosomal proteins. By this method we found that

TABLE I
Crosslinking of IF-3 to Ribosomal Proteins with 2-Iminoethanol

30S r-protein	¹⁴ C content cpm	50S r-protein	¹⁴ C content cpm
S1	N.D.	L1	5
S2	37	L2	369
S3	73	L3	22
S4	121	L4	189
S5	17	L5	305
S6	8	L6	14
S7	76	L7	5
S8	76	L8	3
S9/S11	842	L9	11
S10	129	L10	30
S12	555	L11	54
S13	71	L12	10
S14	76	L13	68
S15	61	L14	88
S16	40	L15	71
S17	N.D.	L16	30
S18	11	L17	511
S19	79	L18	60
S20	127	L19	78
S21	36	L20	36
		L21	82
		L22	114
		L23	18
		L24	28
		L25	10
		L26	13
		L27	119
		L28	142
		L29	2
		L30	0
		L31	2
		L32	1
		L33	0
		L34	N.D.

N.D. = not determined

IF-3 was strongly crosslinked to S9/S11 and S12 in purified 30S subunits and to L2, L5 and L17 in purified 50S subunits (Table I).

These results shown in Table I summarize one of three separate cross-linking experiments. Since S9 and S11 are difficult to resolve in the gel system we have used, there is ambiguity in assigning the IF-3 crosslink to either of these proteins. We favor S11 as the crosslinked protein since

2-iminothiolane requires a reactive sulfhydryl group, which S9 lacks. Another set of proteins (S4, S10, S20, L4, L22, L27 and L28) appears in Table I to contain some radioactivity, and might be considered to be weakly crosslinked to IF-3. The counts seen are, however, barely above background levels, and we do not consider this set of proteins to be cross-linked. Indeed, S10, S20, L4 and L22 do not contain sulfhydryl groups, so we do not see how 2-iminothiolane could crosslink IF-3 to these proteins.

The U.V. difference spectrum observed when IF-3 is added to a ribosome solution at 10 mM Mg^{2+} is shown in Figure 3. A positive peak is observed at approximately 260 nm, with a minimum at approximately 230 nm. The change in absorbance at 260 nm is linearly related to the ribosome concentration, at a constant IF-3/ribosome ratio of 2, over the range of 5 nM - 55 nM, in accord with Beer's Law. The observed effect is in fact very slight, and represents an increase in the absorbance of only about 1% relative to the initial ribosome solution without IF-3. However, the spectrum has been consistently observed using several preparations of both ribosomes and IF-3. Similar titration of ribosomes with bovine serum albumin or lysozyme produces no spectral change.

Concomitant sedimentation velocity centrifugation on solutions having a difference spectrum such as that in Figure 3 show sedimentation profiles

Fig. 3. UV difference spectrum of ribosomes versus ribosomes plus IF-3

This was performed as described in Materials and Methods. The spectrum shown is an average of nine separate determinations. In all cases 1.5 - 2.5 molar equivalents of IF-3 were added to ribosome solutions of a concentration of 43 pmol/ml ($2A_{260}/ml$) in a buffer containing 10 mM Tris-Cl, pH 7.8, 60 mM KCl, and 10 mM Mg^{2+} . After 15 min incubation at 37°, the spectra were recorded at 25°.

identical to those for reference ribosome solutions at the same concentration and ionic conditions, indicating that the observed spectral change is not due to ribosome dissociation. IF-3 may therefore be acting in some other fashion to produce the observed change. At low magnesium concentrations (2.5 mM) the IF-3 used shows its characteristic dissociation activity as judged from sedimentation velocity experiments. Two molar equivalents of IF-3 result in ~75% dissociation of the ribosomes under such ionic conditions.

As shown in Figure 4, the magnitude of the observed difference spectra varies with the amount of IF-3 added until approximately 1 molar equivalent has been added, and is essentially constant after that point. The data are insufficient to calculate a binding constant with any confidence, although the saturation, near a 1:1 molar ratio suggest relatively tight binding. At the concentrations used, a binding constant on the order of 10^8 M^{-1} would produce saturation at that level, assuming the reaction :

DISCUSSION

Our results show that IF-3 may be crosslinked to specific proteins from both 30S and 50S subunits, suggesting that each subunit contains a binding site for the factor. Interaction of IF-3 with the 70S ribosome at a binding site composed of portions of both subunits seems feasible. UV difference spectroscopy provides evidence that such interactions can indeed

Fig. 4. Titration of ribosomes with IF-3

This was performed as described in Materials and Methods.

occur at 10 mM Mg²⁺.

We find strong crosslinking of IF-3 to 30S proteins S9/S11 and S12. These complement the catalog of reported IF-3-30S protein crosslinks previously reported with other crosslinking reagents. By using dimethylsuberimide, Hawley *et al.* (10) crosslinked IF-3 to S12. Heimark *et al.* (19), with dimethylsuberimide or N, N-p-phenylene-diaimide, reported IF-3 crosslinks to S1, S11, S12, S19 and S21. Photochemically induced crosslinks of IF-3 to S7, S11, S12, S18 and S21 were also reported by MacKeen *et al.* (20). In addition, van Duin *et al.* (21), observed an IF-3-S7 crosslink. Taken together, these results suggest that 30S proteins S1, S7, S11, S12, S13, S18, S19 and S21 are in close proximity to the IF-3 binding site. Immune electron microscopy and neutron diffraction studies have localized this set of proteins to a well defined neighborhood in the "platform" region of the 30S subunit (22-24).

We find strong IF-3 crosslinks to 50S proteins L2, L5 and L17. We believe this to be the first report of specific IF-3 crosslinks to 50S proteins. After completion of this work, Schwartz reported photochemically induced crosslinks to L2, L4, L27 and L33 (25). Of particular interest is the recent report of Cover *et al.* (26) on the crosslinking of specific 50S proteins to 30S proteins in the 70S ribosome. Among the reported crosslinks were L2-S12, L2-S11, L5-S13 and L5-S19. Thus, the 50S proteins to which IF-3 may be crosslinked appear to be in proximity with some of the 30S proteins that are near IF-3 in the 30S subunit. This suggests that the 70S ribosome may have a well defined site to accommodate bound IF-3. Previous reports on the binding of IF-3 to 50S subunits indicated an altered susceptibility of proteins L5, L10, L11 and L27 to lactoperoxidase iodination (25). Immune electron microscopy (26) has suggested that L2, L5, L10 and L27 are localized at the interface, and many of these proteins have been crosslinked to one another by using bifunctional crosslinking reagents (29).

The spectroscopic results presented here suggest that, under the conditions used, IF-3 interacts with the 70S ribosome, and alters its conformation. This observation is consistent with previous indications of a direct interaction of IF-3 with the 70S ribosome. IF-3 has, for example, been reported to alter the electrophoretic mobility of 70S particles (30), and to alter their reactivity towards lactoperoxidase (27). Stoichiometric amounts of IF-3 were crosslinked to 70S ribosomes at 18 mM Mg²⁺ with dimethylsuberimide (31), and the association of IF-3

with 70S particles on sucrose density gradients following glutaraldehyde fixation was demonstrated (32). Furthermore, IF-3 was found crosslinked to both 16S and 23S rRNA following periodate oxidation of incubation mixtures of 70S ribosomes and ^{14}C -labeled IF-3, suggesting that regions of both molecules might form a binding site on the 70S particles (33). Taken together, these findings are inconsistent with assertions that IF-3 does not interact with the 70S ribosome, based largely on the results from sucrose density gradient centrifugation (34-37).

Considering IF-3 as a dissociation factor, it could act, in principle, by either of the pathways: (I-II-IV) or (I-III-IV):

The pathways I $\rightarrow$ II $\rightarrow$ IV may be recognized as the "anti-association" mechanism, whereby IF-3 sequesters free 30S subunits, and shifts the equilibrium towards dissociation according to Le Chatlier's principle. Direct interaction of IF-3 with the 70S ribosome is precluded. The results presented here, and those cited above, suggest that such interaction is indeed possible, at least under certain ionic conditions. This leaves still unresolved the mechanism by which IF-3 dissociates the ribosome. Equilibrium results are, however, intrinsically incapable of distinguishing between the two pathways, and it must be emphasized that the choice of pathways is a kinetic problem. Kinetic studies that directly address this question have been recently reported (38-42).

The observed maximum in Figure 3 at 260 nm suggests a perturbation of the rRNA in the ribosome by IF-3, either by the disruption of hydrogen bonds or by the unstacking of single stranded bases. Such a notion is attractive given the evidence that 16S and 23S are involved in the association of the 30S and 50S subunits (43-45). Considering the crosslinking data cited earlier (33) one might speculate that IF-3 is disrupting the interface region, producing the observed alteration in the spectrum. More detailed studies than those offered here are needed to substantiate such ideas, and to elucidate the mechanism by which IF-3 displays such diverse functions.

We wish to express some words of caution concerning our results. First, these results were obtained at 10 mM Mg^{2+} . Since IF-3 function and

Mg²⁺ concentration appear to be synergistic, in ways we do not yet fully understand, the results presented here may not be generalized to other Mg²⁺ concentrations. Second, the reagent used for our crosslinking experiments, 2-iminothiolane, requires the proximity of reactive sulfhydryl groups in order to produce a crosslink. Failure to crosslink indicates only that such a group is not nearby and, thus, proteins close to IF-3 may go undetected. This may explain the variance in reported crosslinks discussed earlier. Finally, under the experimental conditions employed we were unable to use the crosslinking method reported here to study IF-3-70S interactions. The H₂O₂ oxidation steps may promote dissociation of the 70S subunit.

In spite of these drawbacks, the methods and results presented here complement previously reported crosslinking, chemical modification and kinetic studies and should provide useful tools for further studies on IF-3-ribosome interactions.

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