THE MECHANISM OF ACTION OF INITIATION FACTOR 3 IN PROTEIN SYNTHESIS, II. ASSOCIATION OF THE 30S RIBOSOMAL PROTEIN S12 WITH IF-3

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Received October 7,1974

Summary: Dimethylsuberimidate was used to crosslink ^{14}C-labeled chain initiation factor 3 to E. coli 30S particles. The crosslinked ribosomal proteins were analyzed by dodecyl sulfate polyacrylamide gel electrophoresis, and one major radioactive aggregate was found corresponding to a molecular weight of 41,000. Ribosomal protein S12 was identified to be crosslinked to IF-3 by immunological cross-reactivity.

Three proteins involved in the initiation of protein synthesis in E. coli were isolated from the 1.0 M NH_4Cl wash of 30S ribosomal particles (1,2). The precise function of these factors has not been unequivocally described, nor has the site of their action been determined. With the growing catalog of protein-protein interactions in the ribosomes structure, one may be able to gain some insight into the functions of the initiation factors if the ribosomal proteins to which they bind were identified.

Recently, chain initiation factor 3 was shown to bind stoichiometrically to washed 30S particles, the binding being essentially independent of temperature and Mg^{2+} concentration (3-5). This factor was also successfully crosslinked to 30S subunits with the bifunctional imidoester, dimethylsuberimidate (6).

MATERIALS AND METHODS

Preparation of 30S Ribosomal Subunits and ^{14}C-Labeled IF-3. Ribosomes were prepared from freshly grown E. coli MRE600 cells and were washed twice with 1.0 M NH_4Cl. 30S ribosomal subunits were prepared from purified 70S ribosomes according to Traub et al. (7).

The preparation of homogeneous IF-3 was obtained as described elsewhere (8,9). IF-3 was labeled by reductive alkylation with [^{14}C]formaldehyde (55 mCi/mmole, New England Nuclear) according to Pon et al. (4). The specific activity of the ^{14}C-labeled factor was 2000 cpm/μg. Its activity in poly(U) translation (9) remained unchanged after modification.

Crosslinking reaction. Dimethylsuberimidate was prepared according to Davies and Stark (10). [^{14}C]H_3-IF-3 was crosslinked to 30S particles as previously described (6).

Polyacrylamide Gel Electrophoresis. Dodecyl sulfate polyacrylamide gel electrophoresis was performed according to Weber and Osborn (11), with the exception that the samples were applied to the gels directly after treatment with SDS. Under these conditions the molecular weight of purified IF-3 was determined to be approximately 24,000. Gels were sliced for counting into 1.5 mm slices and the counts were extracted according to Basch (12) with TS-1 Tissue Solubilizer (Research Products International, Corp.). The slices were counted in Aquasol (NEN) for 10 min per sample in a Packard Tri-Carb scintillation counter.

Preparation of Antisera and Double Diffusion Plates. Antisera against IF-3 or ribosomal protein S12 were prepared in mice as in previous work (13). Ouchterlony plates were prepared as described (13) with the exception that the agarose was dissolved in 10 mM Tris-HCl, pH 7.5, 600 mM NH_4Cl, 0.5 mM magnesium acetate. The plates were incubated for 24 hr at 30° after which they were washed in saline for 12 hr, dried, and stained with a solution containing 0.1% Coomassie blue, 10% acetic acid and 45% ethanol. They were then destained with 7% acetic acid and 5% methanol.

RESULTS

Binding of [^{14}C]H_3-IF-3 to 30S Subunits under Crosslinking Conditions. Although IF-3 was shown to bind stoichiometrically to 30S subunits in Tris-HCl buffers at pH 7.2 to 7.8 (3-5), an initial study was performed to demonstrate stoichiometric binding of the factor in crosslinking buffer [20 mM N,N-bis(2-hydroxyethyl)glycine (Bicine), pH 8.5, 10 mM magnesium acetate, 100 mM KCl]. As seen in Table I, the binding of IF-3 to 30S subunits approaches 0.85 copy per subunit at an input ratio of about 6 copies of IF-3 per 30S particle. Therefore, it appears that under crosslinking conditions IF-3 binds to one site on the 30S subunit.

Crosslinking of [^{14}C]H_3-IF-3 to 30S Subunits. Three equivalents of [^{14}C]H_3-IF-3 were incubated with 30S subunits in Bicine buffer and were crosslinked with dimethylsuberimidate as previously described (6). The crosslinked complexes, which contain 0.9 copy of IF-3 per 30S subunit, were analyzed by 10% polyacrylamide gel electrophoresis in the presence of SDS. The gels were sliced and

TABLE I

Stoichiometry of binding under crosslinking conditions of $[^{14}C]H_3$-IF-3 to 30S particles

Additions of IF-3 per 55 pmoles of 30S particles		Copies of IF-3 bound per 30S particle
μg	(pmoles)	
7.0	(318)	0.82
3.5	(159)	0.69
1.75	(80)	0.61
0.88	(40)	0.22
0.44	(20)	0.11

The indicated amounts of high salt washed 30S particles and $[^{14}C]H_3$-IF-3 were combined at 0° in a total volume of 45 μl of 0.02 M Bicine, pH 8.5, 0.1 M KCl, 10 mM magnesium acetate, 0.4 mM EDTA, and were incubated 15 min at 37° and then at 0° for 20 min. They were layered on 17 ml 5-25% sucrose gradients in the same buffer and centrifuged for 6 hr at 27,000 rpm in the Spinco SW27 rotor. The gradients were fractionated and counted in Triton X-100 scintillation cocktail.

Under crosslinking conditions, the binding approaches 0.85 copy of IF-3 per 30S particle when the chain initiation factor is present in 6-fold excess over the amount of particles.

counted and one major peak of radioactivity was found with a molecular weight of about 41,000 (Fig. 1). This value corresponds to the sum of the molecular weights of IF-3 and a protein of approximate molecular weight of 17,000. Such a peak was consistently present in all preparations of IF-3-30S complexes which were crosslinked under a variety of reaction conditions, and probably represents the major crosslinked product between IF-3 and the 30S subunit. One of the proteins in this molecular weight range is the 30S ribosomal protein S12 (14).

Double Diffusion Plate to Demonstrate IF-3-S12 Complex. The proteins were prepared from the crosslinked IF-3-30S complexes by the acetic acid method (15). After dialysis against 10 mM Tris-

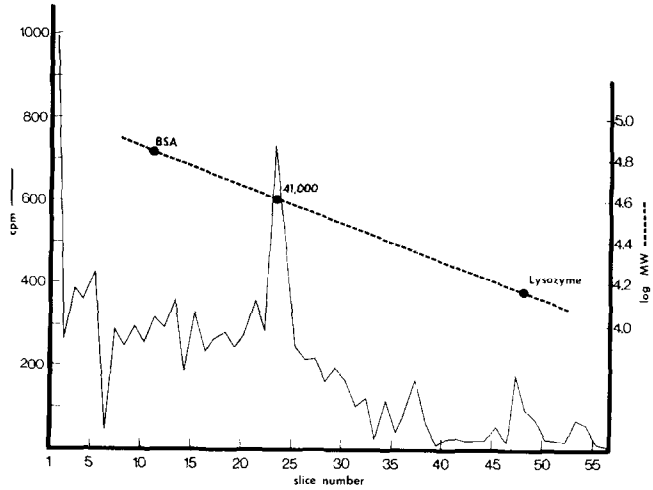

Figure 1. SDS-polyacrylamide gel electrophoresis of 30S ribosomal particles crosslinked with dimethylsuberimidate in the presence of $[^{14}C]H_3$-IF-3. 210 µg of 30S subunits containing 4.1 µg of IF-3 (8,820 cpm) were applied to the gel.

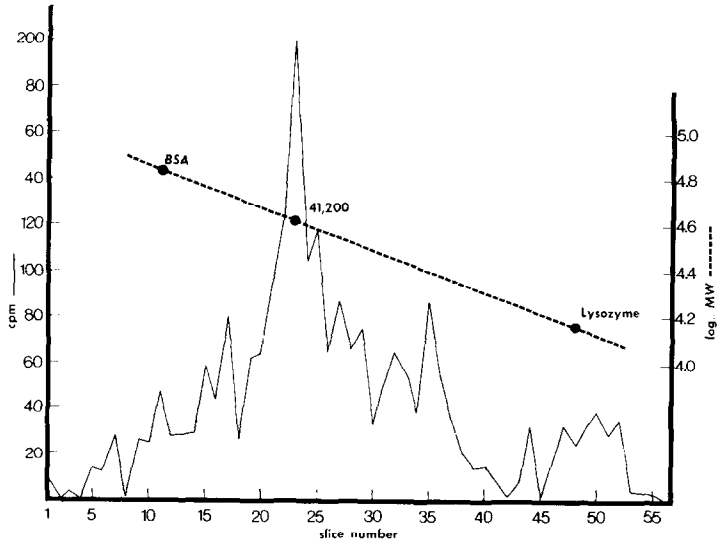

Figure 2. SDS-polyacrylamide gel electrophoresis of crosslinked $[^{14}C]H_3$-IF-3-30S proteins prepared by the acetic acid method (15). 11.5 µg of 30S proteins containing 1.2 µg of IF-3 (2,400 cpm) were applied to the gel.

HCl, pH 7.5, 600 mM NH_4Cl, 0.5 mM magnesium acetate, about two-thirds of the labeled material precipitated. An analytical gel was run on the soluble protein (Fig. 2) which showed that the characteristic peak of radioactivity remained in solution, while most of the larger radioactive aggregates had precipitated. A double dif-

fusion plate was prepared using anti-S12 and anti-IF-3 as adjacent antisera in the outside wells and the soluble ribosomal proteins in the center well. If IF-3 and S12 were covalently crosslinked, they would be expected to behave as a single entity and cross-react with both antisera. Fig. 3 shows a line of identity between anti-S12 and anti-IF-3, indicating that, indeed, the two proteins were crosslinked in the IF-3-30S preparation.

DISCUSSION

One of the current methods used to determine the structure of large protein aggregates is the use of bifunctional protein-specific reagents to crosslink neighboring pairs of proteins (10,16-19). We have attempted to examine the binding site of IF-3 on 30S ribosomes by crosslinking the factor *in situ* with dimethylsuberimidate and determining the molecular weight distribution of the resulting cross linked complexes. We tried to limit the extent of crosslinking so as not to favor the formation of complexes larger than IF-3 and one other protein.

One major radioactive peak with a molecular weight of 41,000

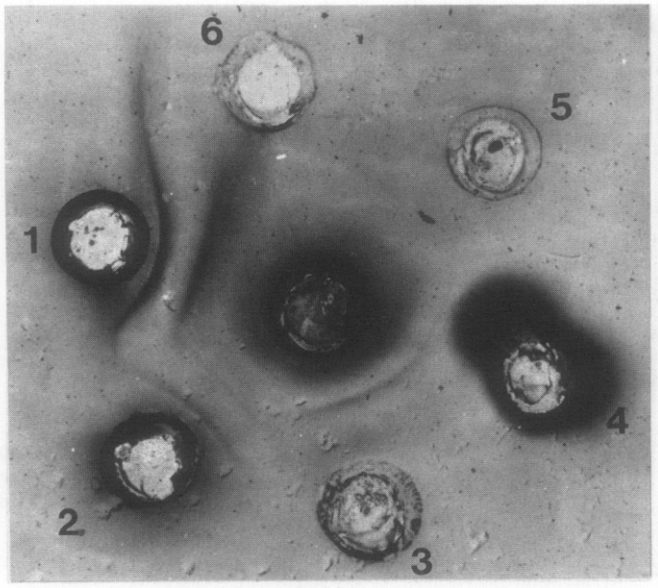

Figure 3. Immunological cross-reactivity. The double diffusion plate was prepared as described under "Materials and Methods". The outside wells contained: 1, 10 µl of anti-IF-3; 2, 10 µl of anti-S12; 3, 2 µl of anti-IF-3 in 8 µl of saline; 4, 2 µl of anti-S12 in 8 µl of saline; 5, 10 µl of pre-immune serum. The center well contained 0.5 µg of crosslinked IF-3 in 10 µl of ribosomal proteins (575 µg/ml).

was found by analysis of crosslinked [^{14}C]H_3-IF-3-30S complexes on SDS-polyacrylamide gel electrophoresis. Ribosomal protein S12 was found to be crosslinked to IF-3 in this aggregate as judged by cross-reactivity between the protein and antisera prepared against IF-3 and S12. There is the possibility that the 41,000 molecular weight peak represented complexes of IF-3 and S11 or S9 as well as the S12 aggregate, but the appropriate analyses for these proteins have yet to be completed.

Ribosomal protein S12 was shown to be involved in the proper functioning of 30S subunits as well as in streptomycin fixation to sensitive 30S particles (20,21). Additional work suggesting the involvement of S12 in initiation of protein synthesis was carried out using ribosomes from streptomycin dependent cells. These ribosomes, which are known to have mutational alterations in S12, bind fMet-tRNA in the presence of poly AUG or T4 mRNA with about one-half the efficiency of streptomycin sensitive ribosomes (22). Initiation factors were also found to compete with streptomycin in binding to both 30S and 70S sensitive particles (23). On the other hand, recent data indicate that monovalent antibodies against S12 do not completely block dihydrostreptomycin binding to 30S subunits (24). In this laboratory our results demonstrate that IF-3 binds to S12 on free 30S particles. This suggests that S12 is directly involved in the initiation event.

Other crosslinks between IF-3 and the 30S subunit are presently under investigation. Preliminary evidence indicates that IF-3 crosslinks to S1 both on the 30S subunit as well as in solution.

ACKNOWLEDGEMENTS

The authors are indebted to Alain Niveleau for the preparation of IF-3. This work was supported by grants from the Medical Research Council of Canada (A. J. W.) and the National Science Foundation (L. I. S.).

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