Hypothesis

THE TOPOGRAPHICAL LOCALIZATION OF IF3 ON *ESCHERICHIA COLI* 30 S RIBOSOMAL SUBUNITS AS A CLUE TO ITS WAY OF FUNCTIONING

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1. Introduction

A problem of considerable interest concerns the topographical localization of the translational initiation factors on the ribosome. This is essential to improve our knowledge of the ribosomal topography and to gain a better insight into the physical and mechanistic aspects of the interactions between the factors and the ribosome. Several protein–RNA and protein–protein crosslinking studies dealt so far with this subject (reviews [1,2]).

Here, we present results obtained on the protein neighborhood of IF3 by use of very mild reaction conditions. Taking into account the functional and structural properties of IF3, its topographical localization on the ribosome and its effect on the properties of other ribosomal proteins, we also present an hypothetical model which attempts to explain how the factor might function.

2. Materials and methods

2.1. *Buffers*

Buffer (A): Tris–HCl (pH 7.7), 10 mM; Mg-acetate, 10 mM; NH_4Cl, 60 mM; 2-mercaptoethanol, 6 mM

Buffer (B): Triethanolamine–HCl (pH 8.5), 50 mM; $MgCl_2$, 5 mM; KCl, 100 mM

Buffer (C): Tris–HCl (pH 7.7), 20 mM; EDTA, 0.5 mM; NH_4Cl, 200 mM; glycerol, 10%; DTT, 1 mM

2.2. *Preparation of ribosomes, ribosomal subunits and factor*

Escherichia coli MRE 600 high-salt washed ribosomes and ribosomal subunits were prepared [3]. For some experiments 70 S 'tight couples' and ribosomal subunits derived from them were prepared essentially as in [4]. Initiation factor IF3 was purified [5], and labelled in vitro by either reaction with *N*-[^{3}H]ethylmaleimide (NEM) or by reductive methylation with [^{14}C]formaldehyde as detailed in [6]. Poly(U)-dependent polyphenylalanine synthetic activity test for 30 S ribosomal subunits was performed as in [7].

2.3. *Crosslinking reaction*

Complexes of [^{3}H]NEM-IF3 with 30 S ribosomal subunits were prepared by incubating, in a 15 ml glass centrifuge tube, the desired amounts of 30 S ribosomal subunits in buffer A with 1.5–2.0 molar-equivalents of the factor at 37°C for 10 min. The samples were then chilled in ice, 0.7 vol. cold ethanol added and mixed. After 10 min in an ice bath the samples were centrifuged to collect the precipitated IF3–30 S complex. The pellets were drained and dried, and sufficient buffer B was added to resuspend the ribosomes to 50 A_{260} units/ml. An equal volume of the same buffer containing 8.5 mg/ml of freshly dissolved dimethylsuberimidate (DMS) (Pierce) was added to the ribosomal solution. The reaction was allowed to proceed at 20°C with constant stirring. At the beginning of the reaction the pH drops and it is necessary to readjust it to ~8.2 by addition of a 1 M solution of triethanolamine (Merck). At the desired times the reaction was stopped by addition of a 4 M NH_4Cl solution to 0.5 M final conc. and by addition of 1 M Tris–HCl (pH 7.2) to lower the pH to ~7.5.

2.4. *Identification of the crosslinked products*

Crosslinked products were identified by electrophoretic analysis and M_r determinations as in fig.1; in addition, immunological identifications by

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Ouchterlony double-diffusion tests using antisera against all individual 30 S ribosomal proteins [29] were kindly made by Miss R. Hasenbank and Dr G. Stöffler.

3. Results

The reaction of 30 S–IF3 complexes with dimethylsuberimidate results in the formation of crosslinked products between IF3 and 30 S ribosomal proteins as well as in the inactivation of the 30 S ribosomal subunits. In preliminary experiments we followed the time course of both appearance of crosslinked products of different M_r-values and of inactivation of the 30 S ribosomal subunits. Thus, we were able to select mild reaction conditions which resulted in minimal loss $\leqslant$20% of the biological activity, as well as in minimal formation of protein aggregates. All protein neighbors of IF3 described here were identified from crosslinked complexes produced under these mild conditions. Furthermore, to have all the ϵ-NH_2 groups of IF3 available for the reaction with DMS and to prevent the possible formation of S–S bridges between IF3 and –SH-containing *r*-proteins, we avoided the use of reductively methylated IF3, but used IF3 labelled in vitro with [^{3}H]NEM.

In fig.1A an electrophoretic analysis of the time course of crosslinking IF3 to 30 S *r*-proteins is presented. At the onset of the reaction (0 time), the only radioactive protein was IF3. As the reaction proceeded, the amount of non-crosslinked IF3 decreased drastically and a number of discrete radioactive bands of M_r higher than IF3 appeared. The amount of these products increased with time of reaction as did their M_r-values. No difference in this crosslinking pattern was observed with different 30 S preparations (including 30 S derived from 'tight couples'). The polyphenylalanine synthetic activity of the 30 S ribosomal subunits was found to be 80% of the unreacted controls after 30 min reaction, but longer incubation times resulted in almost total loss of activity (not shown). The M_r-values of the crosslinked products were determined by comparison with M_r markers (fig.1B) and possible precursor–product relationships between the different crosslinked complexes were analyzed by scanning with a microdensitometer the autoradiographies of several gels similar to that of fig.1A. From these data and from immunological identification, the protein neighbors of IF3 were found to be S11, S13 and S19 (see table 1). Experiments were also carried out to determine whether it was possible to crosslink

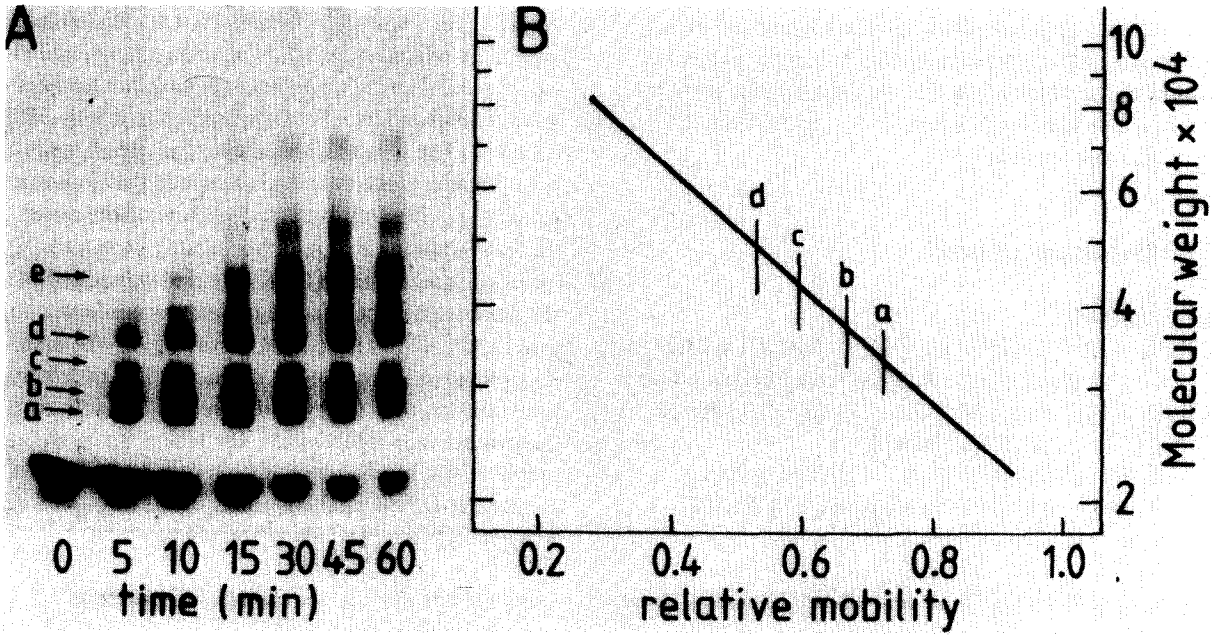

Fig.1. Crosslinking [^{3}H]NEM IF3 to 30 S ribosomal proteins by reaction with dimethylsuberimidate: (A) autoradiography of an SDS gel electrophoresis showing the time course of crosslinking; (B) estimation of M_r-values of the reaction products. The conditions of the experiment are described in section 2.

Table 1
Ribosomal proteins crosslinked to IF3[a]

Electrophoresis[b] band	M_r (determined)	Complex	M_r (exp.)[c]	M_r (exp.)[d]
a	33 500	IF3–S19	35 700	30 994
b	37 500	IF3–S13	37 500	33 663
		IF3–S11	38 100	34 423
c	43 000	(IF3–IF3)	45 200	41 390
d	49 000	(IF3–S19–S13)	50 600	43 962
e	63 000	(IF3–S19–S13–S11)	66 100	57 690

[a] The M_r of IF3 is 22 600 from SDS gel electrophoresis and 20 695 from primary structure [1]
[b] The indicated bands correspond to those seen in fig.1. The first 3 complexes have been identified directly by Ouchterlony double diffusion tests [29]. The existence of the complexes in parentheses is postulated on the basis of their M_r-values and of the immunological data
[c] M_r expected from the M_r determined by SDS gel electrophoresis [30]
[d] M_r expected from the amino acid sequences [31] without taking into account the M_r of DMS

IF3 to 50S as reported in [10]; no crosslinking was seen, however, with 50 S or 70 S ribosomes. A possible crosslinking between IF3 and S7 [11] was also specifically investigated by comparing the crosslinking pattern of IF3 with 30 S subunits from *E. coli* MRE600 (which we routinely used) and from *E. coli* A19 whose S7 is 2600 M_r larger [31]. No difference was seen in the crosslinking pattern of these 2 strains and, therefore, we concluded that no link between IF3 and S7 was formed under our experimental conditions.

The identified topographical neighbors of the ribosome-bound factor have been located by immuno-electron microscopy on the head (S13, S19) and on the platform (large protuberance) (S11) of the ribosomal subunit [12] (fig.2A,B) suggesting that IF3 bridges head and platform making contact with both sites on the side of the 30 S facing the 50 S ribosomal subunit. The localization on the 50 S-side of the 30 S agrees with that proposed in [13], while the localization between head and platform agrees with that postulated in [12] and later found experimentally using direct immuno-electron microscopy of IF3 crosslinked to 30 S by dimethylsuberimidate (fig.2).

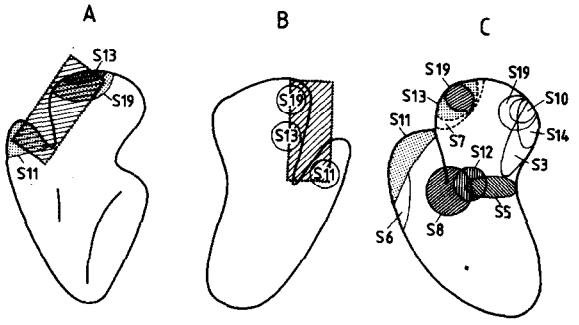

Fig.2. Topographical localization of IF3 on the 30 S ribosomal subunit. Stöffler (A) and Lake and Kahan (B,C) models of 30 S subunits showing the position of the antigenic determinants of the 30 S proteins crosslinked to IF3, the location of IF3 obtained by immuno-electron microscopy (rectangular hatched area), and some of the ribosomal proteins whose reactivity was affected by IF3 presence (see text).

From the topographical localization of ribosome-bound IF3, from the properties acquired by the 30 S ribosomal subunits upon IF3 binding and from what is known concerning the functional and structural properties of the IF3 molecule, we can now try to construct a model to explain a plausible way of functioning of the factor. Any such model must take into account, in addition to that mentioned above, also the following facts:

(i) One of the main functions of IF3 is to promote a several-fold increase in the rates of formation and dissociation of codon–anticodon base-pairing at the 30 S level [14–16].

(ii) The site of codon–anticodon interaction has been localized in the cleft of the 30 S particle [17].

(iii) The presence of IF3 modifies the reactivity of Cys [18] and Tyr [19] residues of several ribosomal proteins: S2, S5, S8 and S21 show increased reactivity, while S11, S12, S17 and S18 show decreased reactivity. All the proteins with increased reactivity and some of the proteins with decreased reactivity (S12, S17, S18) have been localized in the region between head and body (neck) or on the external surface of the platform (protuberance), while S11 which also shows decreased reactivity has been localized on the protuberance, in a region overlapping that of bound IF3 [12] (fig.2c).

(iv) In spite of the fact that static physical methods failed to reveal a large conformational change [20,21], several lines of evidence (alteration of all kinetic parameters of 30 S [14]; alteration of the 30 S rRNA–protein crosslinking pattern [22] and the above-mentioned modified chemical reactivity of several ribosomal proteins in the presence of IF3) indicate that the conformation of the 30 S subunit is changed upon IF3 binding.

(v) Crosslinking of IF3 to rRNA in situ by 2 different methods revealed 2 sites of crosslinking, a major one (80–90%) to the 5′-side of the 16 S rRNA and a minor one (10–20%) to the 3′-end region [23]; these 2 sites are presumably localized in separate regions of the subunit [24].

(vi) Chemical modification experiments [25,27] as well as CD measurements [28] strongly suggest the presence of two active domains in the IF3 molecule.

Taking everything into account, we postulate that, as exemplified in fig.3, the 30 S subunit exists as a number of different conformers separated by small energy barriers in dynamic equilibrium. In the presence of IF3, these energy barriers are lowered, the transitions strongly accelerated and a small alteration of the mean distribution of the conformers takes place. Thus, any static physical measurement (which measures the properties of the average conformer) will fail to reveal a conformational change. On the contrary, the 30 S characteristics which are affected by their dynamic properties (i.e., rate of chemical modifications, functional properties) will be profoundly affected by the presence of the factor. The different 30 S conformers could be characterized by the relative orientation of head and body (or head and platform) with a hinge being located somewhere in the neck region. If, as indicated in fig.3, IF3 contains 2 active sites, A and B, interacting respectively with head and platform (protuberance) of the subunit, the effect of the factor on the conformational dynamics of the 30 S particle could be generated by 2 fluctuating interactions (or a stable and a fluctuating one) resulting from the interplay of attractive and repulsive forces (e.g., electrostatic and hydrophobic). This would result in the relative movement of head and protuberance of the 30 S ribosomal subunits in a sort of a nodding fashion which could influence the rate of association and dissociation of the codon–anticodon complex, the reactivity of those proteins located at the region of the hinge as well as the rate of association of the 30 S with the 50 S subunit.

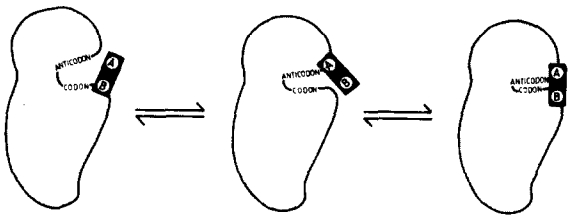

Fig.3. Schematic representation of a possible mechanism of IF3 functioning (see text). The model is based on the existence of three 30 S conformers and two fluctuating IF3 interactions. However, a number of conceptually similar models involving a different number of both 30 S conformers and more or less stable interacting sites could be envisaged.

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