SEPARATION OF TWO FORMS OF IF-3 IN *ESCHERICHIA COLI* BY TWO-DIMENSIONAL GEL ELECTROPHORESIS

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Received 18 May 1977

1. Introduction

Among the three initiation factors which are required by *Escherichia coli* ribosomes for translation of natural mRNA, IF-3 has generally been considered to play a key role. It is required to maintain a pool of free 30 S subunits [1,2] which normally initiate protein biosynthesis (reviewed in [3]). In vitro IF-3 stimulates the translation of natural and synthetic messengers [3,4]. The protein has been located in the neighborhood of 16 S RNA [5] and of several ribosomal proteins that are involved in the initiation step [6]. Earlier studies also implicated a role for it in transcription [7].

Previous reports [8–10] suggested that several forms of IF-3 may exist in *E. coli*, and two distinct IF-3 species were purified by Lee-Huang and Ochoa [11,12]. The two species differed from each other in mRNA discrimination, molecular weight, isoelectric point and chromatographic behavior [12]. Other workers could isolate only a single species of IF-3 from normal or T4 phage-infected *E. coli* [4,13].

Recently we have been studying by two-dimensional gel electrophoresis the proteins specifically associated with native 30 S ribosomal particles. About 15% of the total ribosomes in *E. coli* extracts (in 0.01 M Mg^{2+}) exist as native subunits. The native 30 S subunits have earlier been shown to contain initiation [14] and dissociation factor [15] activities and our experiments (unpublished) show additional proteins, present stoichiometrically.

During our study we obtained purified factor preparations from several laboratories for identification in two-dimensional gel systems [16,17]. When these preparations were analyzed, it became clear that different laboratories have purified two different molecular forms of IF-3. Therefore we examined ribosomes and native 30 S subunits from different strains of *E. coli* and the results presented below show that *E. coli* contain at least two forms of IF-3. Their primary structure is given in the accompanying paper [18].

2. Materials and methods

IF-3 preparations were obtained from Drs L. Bosch, C. Gualerzi, S. Ochoa, A. J. Wahba and H. G. Wittmann.

E. coli (MRE600 and A19 strains) were grown in L-broth at 37°C in either 5 liter flasks or a 50 liter fermenter. Measurements of growth, harvesting etc. were as described [19]. Cells were ruptured by grinding with alumina or by subjecting a suspension to three 15 s pulses of ultrasonic waves (MSE, 7100) or to two passages in a French Press at 10 000 lb/in^2. The buffer (0.01 M Tris–HCl, pH 7.6, 0.05 M KCl, 0.01 M Mg acetate, 0.006 M 2-mercaptoethanol) contained 1 μg/ml DNAase. The extract was centrifuged at 30 000 × *g* for 30 min. Native subunits and 70 S ribosomes were separated by sucrose gradient centrifugation (in above buffer) in Beckman, type Ti-15 zonal or SW 27.1 rotors. The peak regions were pooled and ribosomal particles isolated by overnight pelleting at 150 000 × *g*.

Proteins were extracted from ribosomes by 67% acetic acid [20] and electrophoresed as described elsewhere [16,17]. Ribosome concentrations were estimated from absorbance at 260 nm and protein concentrations by the method of Lowry et al. [21] with bovine serum albumin as standard.

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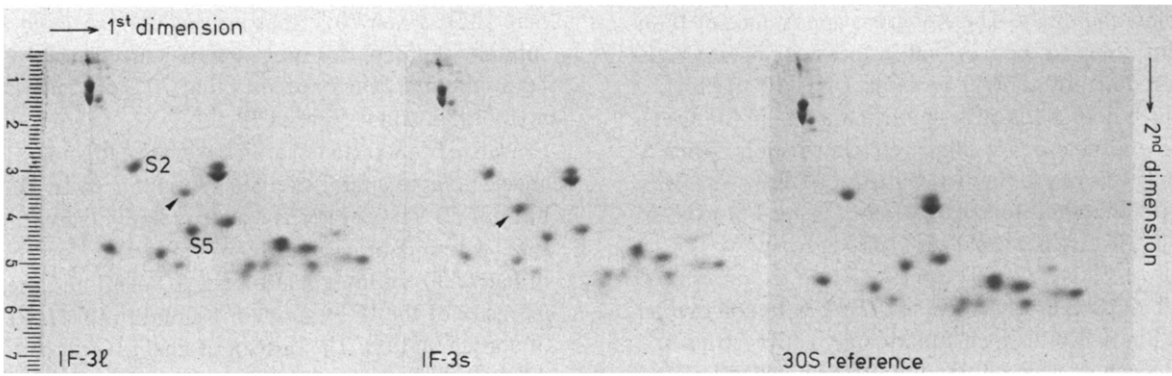

Fig.1. Two-dimensional gel patterns of the two forms of IF-3 coelectrophoresed with 30 S ribosomal proteins as reference. About 2 μg purified IF-3 was mixed with 30 μg 30 S ribosomal proteins for coelectrophoresis [16]; the third pattern is of the reference proteins alone. Purified preparations given by Drs Bosch and Ochoa corresponded to IF-3s, while those from Drs Gualerzi and Wahba correspond to IF-3ℓ.

3. Results

3.1. *Positions of purified IF-3*

The different purified IF-3 preparations were coelectrophoresed [16] with 30 S ribosomal proteins to determine the position of IF-3 in relation to the latter. In each case a single spot which did not overlap with any 30 S ribosomal protein appeared. Closer inspection revealed that this spot did not occupy a single invariant position, but one or the other of two distinct positions. Figure 1 shows the two patterns and that for reference 30 S subunits. The distinct positions of IF-3 (indicated by arrow) are clearly seen when the spots of IF-3, S2 and S5 are joined by imaginary lines. In one case (IF-3s) the three spots lie in a straight line while in the other they lie at the corners of a triangle. We have designated the two forms IF-3ℓ and IF-3s.

The two forms of IF-3 were also run in the standard two-dimensional gel system [17] and the results are shown in fig.2. The position of IF-3ℓ is exactly below protein L1 whereas that of IF-3s is

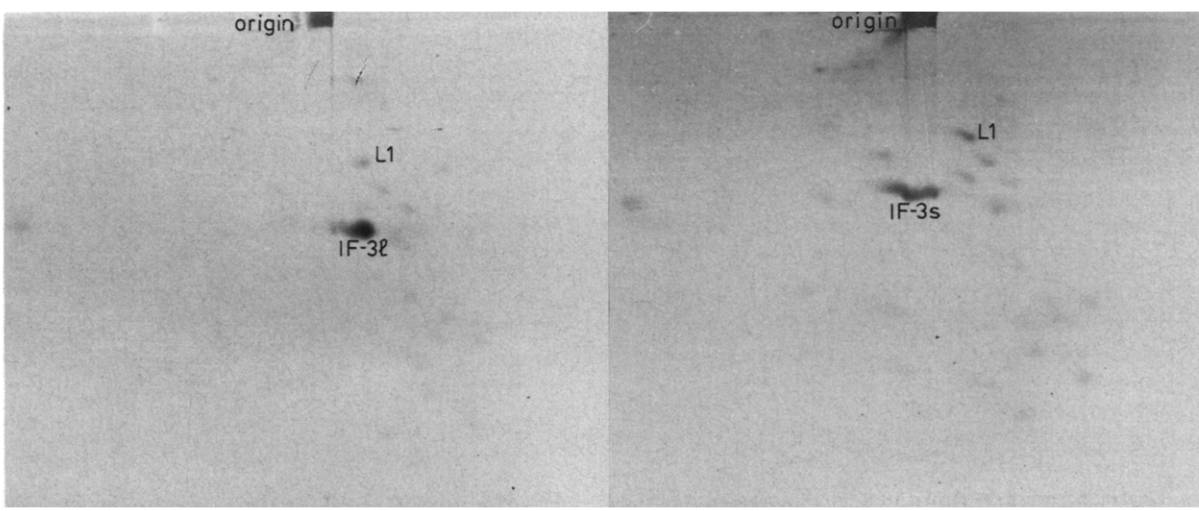

Fig.2. Positions of IF-3ℓ and IF-3s after two-dimensional electrophoresis according to Kaltschmidt and Wittmann [17].

below the origin. The elongated appearance of IF-3s spot is due to the fact that it does not migrate in the first dimension; other proteins, e.g., S10 and L21, which do not (or only slightly) migrate in the first dimension also give similar elongated spots. When IF-3s was run in the first dimension under somewhat different conditions, i.e., pH 9.6, instead of pH 8.6 [17], it gave a normal spot (not shown).

3.2. *Occurrence of IF-3s and IF-3ℓ in* E. coli *extract*

Since two distinct, functionally active forms of IF-3 can be isolated, the important biochemical question is whether these two forms actually occur in *E. coli* or whether they are produced merely during the purification of IF-3.

In order to try to answer this question we prepared extracts of *E. coli* and subjected them immediately to fractionation by zonal centrifugation. Four cuts, i.e., 70 S ribosomes, native 50 S and 30 S subunits and post-ribosomal supernatant, were collected and extracted with 67% acetic acid and the proteins electrophoresed. Figure 3 shows the patterns obtained for native 30 S subunits and 70 S ribosomes. In the native 30 S pattern are seen spots which correspond to IF-3s and IF-3ℓ: when purified IF-3s and IF-3ℓ were co-electrophoresed with native 30 S proteins they comigrated with these spots.

In the pattern of 70 S ribosomes, no spot corresponding to IF-3ℓ could be seen. There is a strong spot at about the position of IF-3s, but we have earlier shown that the ribosomal protein L5 migrates here [22]. When 70 S ribosomes were dissociated and subunits isolated, this spot migrated entirely with 50 S subunits. Hence we infer that 70 S particles do not contain either form of IF-3.

Native 50 S particles, which we isolated and analyzed, gave a faint spot corresponding to IF-3ℓ, but our 50 S preparations were contaminated with small amounts of native 30 S particles. The degree of native 30 S contamination was 10–15% and the intensity of the IF-3ℓ spot correspond to that level of contamination. The post-ribosomal supernatant did not show any spot in this region as was noted earlier [16].

In other experiments *E. coli* cells were ruptured by different procedures, i.e., sonication, passage through French Press, and ribosomal particles isolated. In all cases the two forms of IF-3 were always found specifically associated with native 30 S particles. Native 30 S particles isolated from different strains of *E. coli* (MRF600, A19 and CP78) also showed spots corresponding to IF-3s and IF-3ℓ. Their relative intensities were approximately in the ratio IF-3ℓ : IF-3s = 4 : 1 (by visual estimation) in MRE600 an A19 strains. This ratio was the same whether we used frozen cells or freshly harvested cells and processed them as fast as is practicable.

IF-3 activity is present in ribosomal pellets obtained by high speed centrifugation and can be washed off by 1 M NH_4Cl, but not 0.5 M NH_4Cl [3]. Spots corresponding to both IF-3s and IF-3ℓ are clearly seen in electropherograms of 1 M NH_4Cl

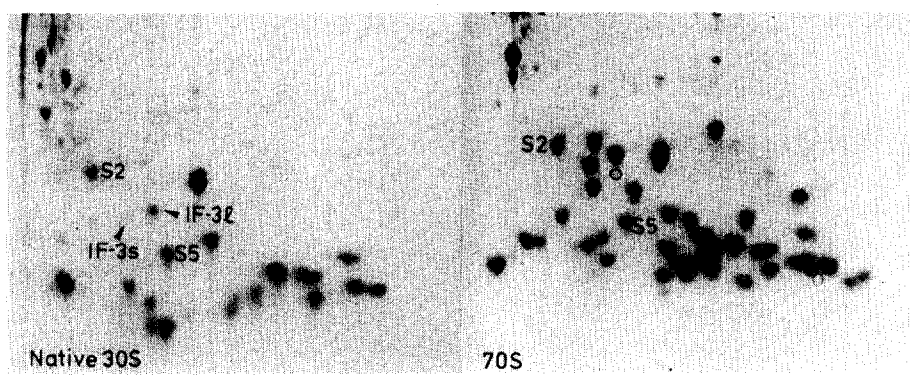

Fig.3. Electropherogram of native 30 S subunit proteins showing the presence of two IF-3 species, and that of gradient purified 70 S ribosomes showing their absence. The prominent spot in the position of IF-3s in 70 S ribosomes is due to L5 [22]; the circle indicates the expected position of IF-3ℓ.

wash proteins (data not shown). A faint spot of IF-3ℓ is seen also in the total proteins extracted from unwashed ribosomes (IF-3s is obscured by L5, as noted), but not in that extracted from 1 M NH_4Cl washed ribosomes.

4. Discussion

As noted in the Introduction, experimental evidence relating to multiple species of IF-3 has had a curious history [4,8–13]. We have given above our evidence for the existence of two molecular forms of IF-3. In *E. coli* cell extracts both forms are associated only with the native 30 S subunits and the amount and relative proportion of the two forms were independent of the method of cell lysis and the nature of the bacterial strain used.

As is apparent from their electrophoretic behavior the two forms of IF-3 reported in this paper differ slightly in molecular weight and isoelectric point. They are separated into two distinct bands in the sodium-dodecylsulphate gel system of Laemmli ([23] data not shown). The form IF-3ℓ has a higher molecular weight and a more basic isoelectric point than IF-3s.

The molecular weights and isoelectric points of the two forms of IF-3 reported by Lee-Huang and Ochoa [12] were 23 500 and 8.3 for IF-3α and 21 500 and 8.4 for IF-3β. The possible correspondence between these two and the ones reported in this paper have not been determined since purified α and β forms were not available. A small anomaly should be pointed out, however: in the case of the α and β forms the lower molecular weight form is more basic while we find our higher molecular weight form as the more basic protein.

The amino acid sequences of IF-3ℓ and IF-3s have been determined and are given in the accompanying paper [18]. The two proteins differ in primary structure only in terms of a hexapeptide at the N-terminal region. It is therefore likely that the two forms are both products of the same gene. In preliminary experiments an enzyme activity which converts IF-3ℓ to IF-3s has been detected (unpublished).

The two forms of IF-3 obtained from different laboratories were both active in phage RNA-directed protein synthesis and in ribosome dissociation [4,24–26]. Both forms were also active in releasing nonspecifically bound amino acyl tRNA from 30 S subunits ([27], Gualerzi, personal communication). Lee-Huang and Ochoa noted [12] that their IF-3α is more active than IF-3β in phage MS2 RNA-directed protein synthesis whilst both forms were equally active in ribosome dissociation. Further experiments are necessary to find the functional differences between the two forms reported in this paper. It has been reported that IF-3 activity in the cell is particularly unstable and is strikingly diminished in the stationary phase [28] or upon chloramphenicol treatment [29,30] and presently we are studying these metabolic aspects of stability and interconversion of IF-3ℓ and IF-3s to find the possible physiological significance for their existence.

Acknowledgements

We thank those (named in the text) who generously provided purified IF-3 and Professor H. G. Wittmann for discussions, comments and other help.

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