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The NH_2-terminal cleavage of *Escherichia coli* translational initiation factor IF3

A mechanism to control the intracellular level of the factor?

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A short form of *Escherichia coli* translational initiation factor IF3, repeatedly found both in vivo and in vitro, lacking the positively charged N-terminal hexapeptide has been produced by mild trypsinization. The properties of this short form of IF3 have been studied. Compared to the long native form of the factor, the shortened IF3 displays a markedly decreased thermal stability and affinity for the 30 S ribosomal subunit, as well as a reduced biological activity in protein synthesis. Following the loss of the N-terminal hexapeptide, a second peptide bond (Lys-90–Val-91) becomes easily accessible to proteolytic attack suggesting that formation of the short IF3 may be the first step in the physiological degradation of the factor.

Protein synthesis; Initiation factor; Thermal stability; Ribosome; Proteolysis

1. INTRODUCTION

Initiation factor IF3 is one of the three factors essential for translational initiation (review [1,2]). Earlier characterization of *E. coli* IF3 has shown that two forms of this factor may exist: a long one (native form) consisting of 181 amino acid residues and a shortened one lacking the first six amino acid residues [3,4]. Since three positive charges are lost with the N-terminal hexapeptide, native and short IF3 can be easily separated by ion-exchange chromatography, 2-dimensional electrophoresis and, as will be shown here, also by one-dimensional electrophoresis on polyacrylamide gels containing SDS.

Under conditions of IF3 overproduction, massive formation of short IF3 deriving from the cleavage of the native form has been observed [5,6]. Cleavage of native IF3, however, it not restricted to cells overproducing IF3, since, when IF3 preparations from wild type cells derived from different laboratories were compared, some factor preparations were found to be primarily of the short form [4].

To understand if the N-terminal cleavage of IF3 may have any physiological significance, comparison of the properties of the two forms of the factor is necessary.

In the present paper, we show that short IF3 can be produced in vitro by limited trypsinization and that it displays reduced affinity for the 30 S ribosomal subunit, reduced biological activity and decreased thermal stability. Following the cleavage of the N-terminal hexapeptide, a second cleavage site becomes exposed, suggesting that 'shortening' of IF3 may represent the first step in the physiological degradation of the factor.

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2. MATERIALS AND METHODS

2.1. *Buffers*

Buffer A: 30 mM Tris-HCl (pH 7.8); 200 mM NH_4Cl; 0.1 mM NaEDTA; 0.5 mM dithiothreitol. Buffer B: 20 mM Tris-HCl (pH 7.2); 25 mM NH_4Cl; 0.1 mM NaEDTA; 0.5 mM dithiothreitol; 10% glycerol. Buffer C: 20 mM Tris-HCl (pH 7.2); 200 mM NH_4Cl; 6 mM Mg acetate; 0.2 mM spermine; 0.1 mM NaEDTA; 5 mM β-mercaptoethanol; 10% glycerol. Buffer D: 30 mM Tris-HCl (pH 7.3); 50 mM NH_4Cl; 15 mM Mg acetate; 2 mM β-mercaptoethanol. Initiation factor IF3 was purified to electrophoretic homogeneity from *E. coli* MRE600 cells following the published procedure [7]. Protein labelling in vitro by *N*-[^{3}H]-ethylmaleimide (NEM) or by radioactive methylation with [^{14}C]formaldehyde was performed as described [8].

2.2. *Preparative limited trypsinization*

20 μg TPCK-treated trypsin (Merck) were added to 13 mg of purified *E. coli* IF3 in 10 ml buffer A. After 30 min incubation in an ice-bath, the digestion was stopped by addition of 1 mg soyabean trypsin inhibitor. Native and short IF3 were purified from the reaction mixture by chromatography on a phosphocellulose column (1 × 15 cm) equilibrated in buffer B. Short and long IF3 were eluted with a 500 ml linear gradient (0.1–0.6 M NH_4Cl). Further purification of the two forms of IF3 was obtained by gel filtration on a Sephadex G-75 column equilibrated with buffer B containing 0.2 M NH_4Cl. The activity of IF3 was tested by the quantitative procedure described in [9] or from the capacity of IF3 to promote the MS2 RNA-dependent incorporation of radioactive valine into bacteriophage MS2 coat protein [10]. Binding of IF3 to ribosomal subunits was measured in 175 μl buffer C by use of the Beckman Airfuge essentially as described [11]. Each reaction mixture contained *E. coli* 30 S ribosomal subunits (109 pmol), 170 pmol [^{14}C]IF3 (2595 cpm/pmol) and the amounts of either short or native unlabelled IF3 as indicated in fig.3B. After 5 min incubation at 37°C and after centrifugation at 100 000 rpm for 60 min at an actual temperature of ~11°C, 75 μl of the supernatant were quickly withdrawn for determination of the radioactivity due to unbound IF3. Crosslinking of [^{3}H]NEM-labelled IF3 to 30 S ribosomal subunits with dimethylsuberimidate (DMS) and electrophoretic analysis of the products was carried out as described [12].

3. RESULTS

Limited trypsin digestion converts IF3 to a shorter form migrating slightly ahead of the native factor in SDS-containing polyacrylamide gels (fig.1B) and to a smaller product whose size corresponds to approximately half that of the native molecule (not shown). The NH_2-terminal sequences determined for these digestion products are presented in table 1. The short IF3 was found to begin at residue 7 while the smaller product consisted of an approximately equal mixture of two

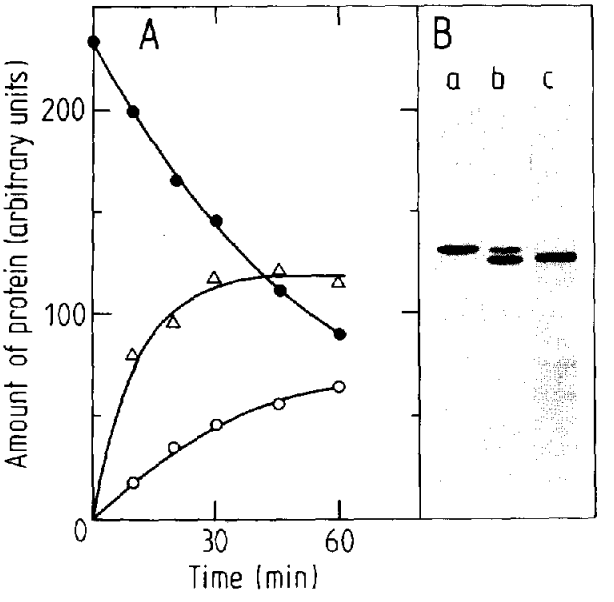
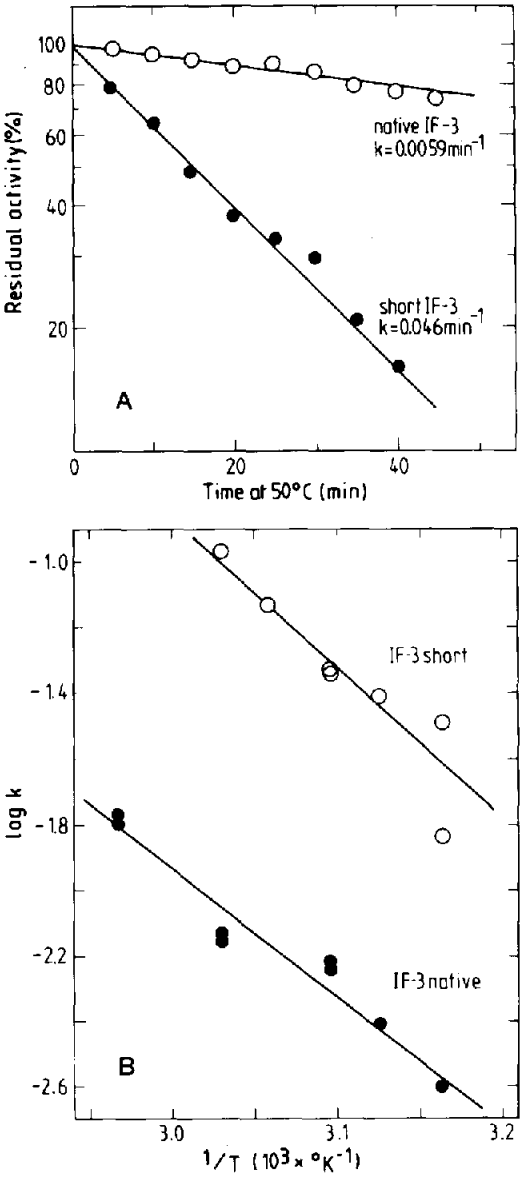


Fig.1. Formation and electrophoretic separation of the short form of IF3. (A) Time course of hydrolysis of IF3 by trypsin. Native IF3 was incubated with trypsin as described in section 2, but on a 200-fold reduced scale. At the indicated times samples were withdrawn for the electrophoretic analysis of the products which were quantified by densitometric scanning of the Coomassie blue-stained gels. (●) Native IF3; (△) short IF3; (○) 'half molecules' generated by cleavage of the Lys-90–Val-91 peptide bond. (B) Separation of native from short IF3 by an SDS-containing 12.5% polyacrylamide gel electrophoresis. (a) Native IF3; (b) native plus short IF3; (c) short IF3.

Table 1
NH_2-terminal amino acid sequence of the limited trypsinization products of IF3

Product	Sequence determined[a]	Position of 1st amino acid in the primary structure[b]
IF3 'short'	Val-Glu-Thr-Ala...	7 →
IF3 'half molecules'	Val-Glu-Thr-Ala...	7 →
	Val-Ile-Gln-Val...	91 →

[a] The amino acid sequence was determined manually as described by Chang et al. [13]
[b] The positions refer to the primary structure presented in [3]



peptides, beginning at residues 7 and 91 and presumably (the C-termini were not determined) ending at residues 90 and 181, respectively.

The appearance of the short form of the factor accompanies the disappearance of the native molecule, while the two half molecules appear later (fig.1A). This finding and the fact that no material of the size of the half molecule was found with the same N-terminus as the native factor suggest that cleavage of the first bond (Arg-6–Val-7) precedes and allows (or at least facilitates) the cleavage of the second one (Lys-90–Val-91).

To investigate the properties of the short form of IF3, the limited proteolysis was scaled up and both native and short IF3 were purified to homogeneity (fig.1B).

A striking difference between the two forms of the factor was found when their rates of heat inactivation were compared. As seen in fig.2A, at 50°C the short form was inactivated nearly one order of magnitude faster than the native one. Similar results were also obtained at other temperatures and the Arrhenius plots of these data yielded two straight and nearly parallel lines for the inactivation of native and short IF3 (fig.2B). This implies that the inactivation of both forms occurs via a single rate-limiting step characterized by approx-

Fig.2. Heat inactivation of native and short IF3. (A) Native (○) or short (●) IF3 (10 μg/ml) were incubated at the indicated temperatures in 50 mM K phosphate buffer, pH 6.9. At the indicated times aliquots were withdrawn and rapidly mixed with 5 ml buffer D on ice. The activity of IF3 was then measured as described in section 2. (B) Arrhenius plot of the heat inactivation of native (●) and short (○) IF3.

imately equal activation energies but different Arrhenius constants (A). This result suggests that the inactivation of the two types of molecules may follow the same (or a very similar) pathway, and it entails complex interactions involving competition between protein-protein and protein-water bonds which are affected by the removal of the N-terminal hexapeptide. That the removal of a short N-terminal peptide could have such a drastic effect on the thermal stability of a protein is not entirely surprising, since 'minor' alterations of the primary structure (e.g. the replacement of a single amino acid) have been found to have profound effects on thermal stability [14–17].

Circular dichroism (CD) and 400 MHz ^{1}H-NMR spectra of native and short IF3 were recorded. With the exception of the lack of the resonance due to the terminal N-methylmethionine in the NMR spectrum of short IF3, neither type of spectroscopy revealed any major difference between the two forms, indicating that the loss of the hexapeptide does not result in a gross alteration of either the secondary or tertiary structure of the factor.

The activity of short and native IF3 in binding to 30 S ribosomal subunits and in their capacity to stimulate MS2-dependent valine incorporation into MS2 phage coat protein were compared. To have a rigorous measure of the amount of long and short IF3 bound to the 30 S ribosomal subunit, we produced short and native IF3 having exactly the same specific radioactivity by modification of the single Cys residue of the protein with N-[^{3}H]ethylmaleimide [8] followed by limited trypsin digestion. Thus, a mixture of [^{3}H]NEM-labelled short and native IF3 was incubated with 30 S ribosomal subunits and the binding analyzed by sucrose density gradient centrifugation. After addition of carrier short and native IF3, trichloroacetic acid precipitation and elec-

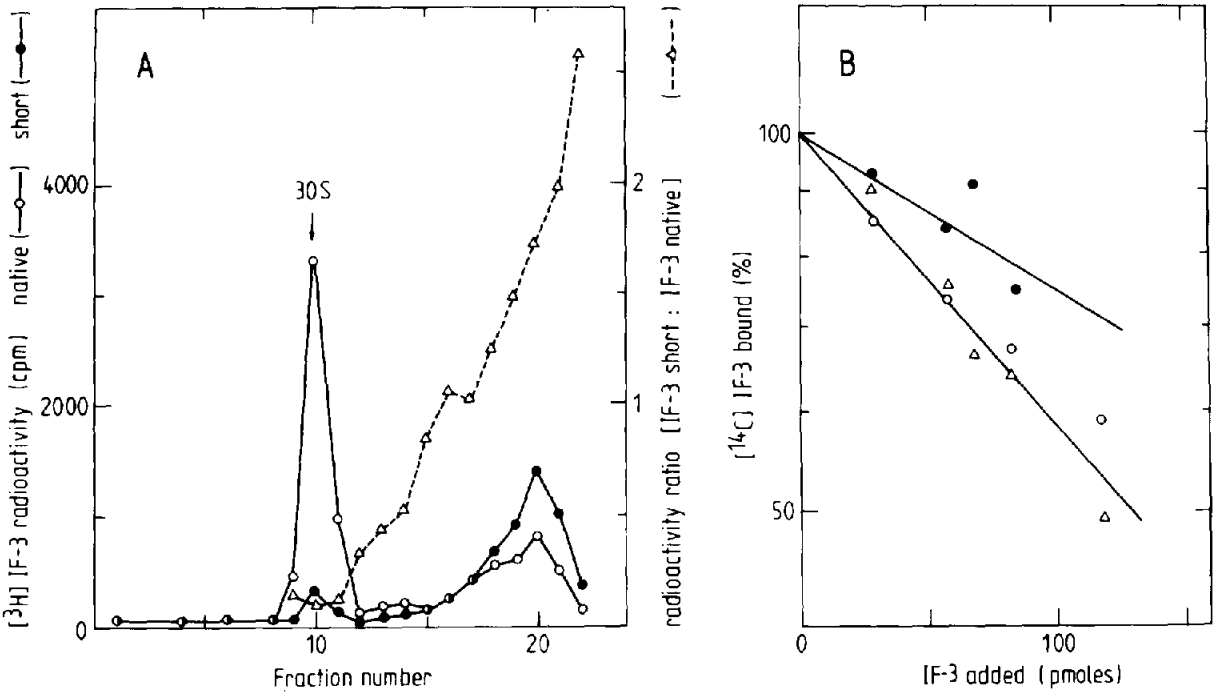


Fig.3. Comparative activity of native and short IF3 in binding to the 30 S ribosomal subunits. (A) The arrow indicates the position of the A_{260} absorbance peak of the 30 S ribosomal subunits. Fraction number refers to the sucrose density gradient fractions. Radioactivity found in the electrophoretically resolved band corresponding to native (○) and short (●) [^{3}H]NEM IF3, (△) radioactivity ratio IF3 short : IF3 native. (B) Competition between [^{14}C]IF3 and non-radioactive native (△) or short (●) IF3 for binding to 30 S ribosomal subunits measured by Airfuge centrifugation as described in section 2. The theoretical competition between [^{14}C]IF3 and IF3 molecules having the same affinity for the 30 S subunit is also shown (○).

trophoretic analysis on SDS-containing polyacrylamide gels, the radioactive material present in each gradient fraction was determined. As seen in fig.3A, the radioactivity found in association with the 30 S ribosomal subunit corresponds almost exclusively to the native form of IF3 while the short form is prevalent in the unbound material at the top of the gradient. The reduced affinity of short IF3 for the 30 S ribosomal subunit is also illustrated by the ratio between radioactive short and native IF3 in each gradient fraction.

Independent evidence for the reduced affinity of short IF3 for the 30 S ribosomal subunit was obtained in an experiment in which increasing amounts of either native or short IF3 were used in competition with native in vitro-labelled IF3 for binding to 30 S subunits. As seen in fig.3B, native IF3 is substantially more efficient than the short form in chasing radioactive IF3 off the 30 S ribosomal subunit. The reduced affinity of the short form of IF3 for ribosomes is also reflected by its diminished efficiency in stimulating MS2 RNA-dependent protein synthesis. Although bacteriophage MS2 coat protein was found to be the main product in both cases (not shown), a larger amount of short IF3 is needed to stimulate the in vitro translation (fig.4).

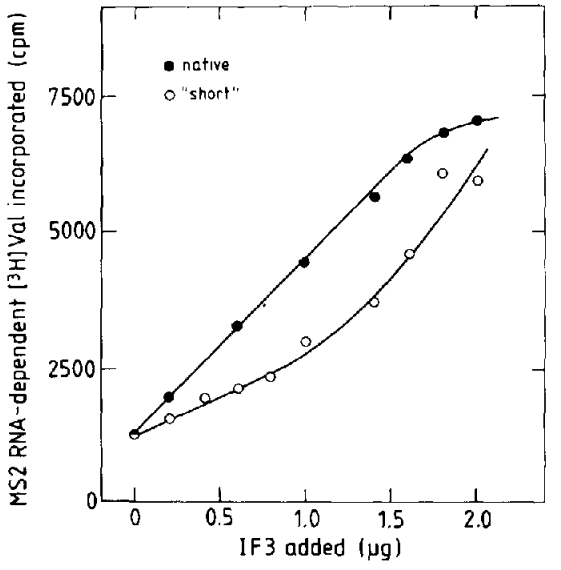


Fig.4. IF3-dependent MS2-RNA-directed protein synthesis. Incorporation of [^{14}C]valine into acid-insoluble product in a cell-free system [10] as a function of increasing amounts of native (●) or short (○) IF3.

Upon binding to the 30 S ribosomal subunit, IF3 can be crosslinked by DMS to ribosomal proteins S11, S13 and S19 [12]. Since this crosslinking reaction involves lysine residues and since the native to short transition entails the loss of two (out of 20) lysines belonging to what is likely to be a flexible and easily accessible end of the protein [18–20], the comparison of the crosslinked products obtained with native and short IF3 could perhaps provide further information on the localization of IF3 on the ribosome by orienting the factor with respect to the ribosomal proteins. Taking into account that short IF3, as well as its crosslinked pro-

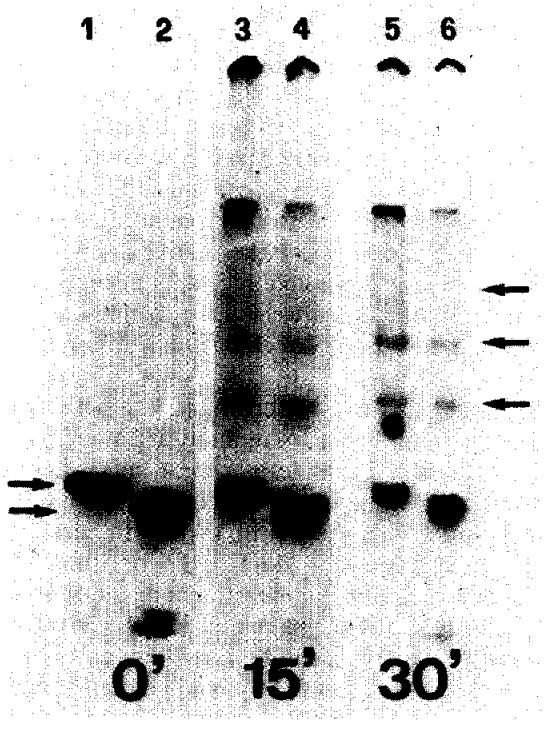


Fig.5. Comparison of the crosslinked products between native and short IF3 and *E. coli* ribosomal proteins. Native and short [^{3}H]NEM-labelled IF3 was bound to the 30 S ribosomal subunits and subjected to crosslinking with dimethylsuberimidate following the published procedure [12]. The figure presents an autoradiography of the crosslinked products electrophoretically separated after the indicated times of crosslinking. Lanes: 1, 3, 5, native IF3; 2, 4, 6, short IF3. The arrows indicate from bottom to top: short IF3; native IF3; IF3-S13 and IF3-S11; IF3-S19-S13; IF3-S19-S13-S11. The identification of the crosslinked products is according to [12].

ducts, migrate slightly ahead of the corresponding ones of the native molecule, we found that the same products are obtained with the two forms of the factor (fig.5). This indicates that the two lysines in the N-terminal hexapeptide are not involved in the crosslinking reaction between IF3 and the above-mentioned ribosomal proteins.

4. DISCUSSION

We have shown here that the Arg-6–Val-7 peptide bond of IF3 is very sensitive to proteolytic attack in vitro resulting in the formation of a shortened form of the factor indistinguishable from that sometimes produced and found in vivo. This cleavage can occur upon addition to native IF3 of minute amounts of trypsin, as in the experiments presented here, or as a result of a trypsin-like activity in *E. coli* cells and cell extracts. In the light of the reduced affinity of the short form of IF3 for the 30 S ribosomal subunit and of its reduced activity in protein synthesis, it seems likely that working with either native or short IF3 may have been responsible for some discrepancies obtained in the past between different laboratories (e.g., the timing of IF3 release from ribosomes, cf. [21]).

It is likely that the reduced biological activity of short IF3 stems from its reduced affinity for the 30 S subunits since, as seen in fig.4, increasing its concentration eventually leads to the same level of activity seen with the native factor. In turn, the reduced affinity of short IF3 for the 30 S ribosomal subunit could be either a direct or an indirect consequence of the removal of the positively charged N-terminal peptide. Even though a selective immobilization of the N-terminal methylmethionine of IF3 by binding of 30 S ribosomal subunits was observed by ^{1}H-NMR spectroscopy [20], there is no evidence for a direct participation of the N-terminal peptide of IF3 in the interaction with the ribosomes. In contrast, even though in a previous work, we have shown that the 30 S ribosomal subunit can protect Lys-2 and -5 from chemical modification with pyridoxal phosphate, we concluded that these residues are not essential for the binding of IF3 to the ribosome [18]. The participation of the N-terminal peptide of IF3 in the interaction with ribosomes also seems unlikely in the light of the fact that the N-terminal sequences of *E. coli* and *Bacillus stearothermophilus* IF3 are different [22] although these factors are interchangeable in binding to the 30 S ribosomal subunits. Thus, the reduced affinity of short IF3 for the 30 S ribosomal subunit is probably an indirect effect possibly due to a slight conformational alteration of the protein. The decrease in thermal stability and the opening of a second cleavage site between Lys-90 and Val-91 in the short IF3 seem to support this premise.

Since the native to short transition occurs massively in cell extracts from strains overproducing IF3 in which considerable amounts of the factor are likely to be ribosome-unbound, and since the first cleavage reduces the affinity of the factor for the ribosomes and exposes a second cleavage site, it is tempting to speculate that the proteolytic removal of the N-terminal peptide of IF3 may play a role in maintaining a constant IF3 : 30 S ratio in the cells.

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