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Proteolysis of *Bacillus stearothermophilus* IF2 and specific protection by GTP

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Translation initiation factor IF2 from *Bacillus stearothermophilus* (741 amino acids, M_r = 82 043) was subjected to trypsinolysis alone or in the presence of GTP. Following electroblotting and automated amino acid sequencing of the resulting peptides, the location and the sequential order of the main cleavage sites were identified. Trypsinolysis of IF2 ultimately generates two compact domains: a 24.5 kDa C-terminal fragment and a 40 kDa G-fragment which is obtained only in the presence of GTP which strongly protects a cleavage site within the GTP binding domain.

Translation; Initiation factor; Thermophilic G-protein; GTP-binding; Protein domain

1. INTRODUCTION

Initiation factor IF2 is the largest of the three proteins required for initiation of protein synthesis in bacteria. The function and the genetics of this factor have recently been reviewed [1-3]. For its function IF2 must interact with the 30 S and 50 S ribosomal subunits as well as with GTP and fMet-tRNA. It has been reported that the interaction of IF2 with GTP or GDP protects the molecule against digestion with three different proteolytic enzymes yielding fragments of similar size [4]. These results indicated the existence of compact structural domains in IF2 and suggested that the interaction of the factor with guanosine nucleotides triggers a conformational change in the protein. The latter conclusion was also supported by the finding that the interaction with GTP favors dipole formation in the IF2 molecules [5].

In the present paper, we have analyzed the pattern of trypsin digestion of *Bacillus stearothermophilus* IF2 in the presence and absence of GTP and determined the sequential order and the main sites of proteolysis. We have identified a peptide bond protected by GTP and identified another one whose accessibility is increased by this ligand.

2. MATERIALS AND METHODS

B. stearothermophilus IF2 was prepared from *E. coli* K12ΔH1Δtrp transformed with pPLc2833 carrying the *B. stearothermophilus infB* gene as previously described [6]. Purification of the factor followed essentially the published procedure [7].

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Trypsinolysis was carried out at 37°C in 210 μl reaction mixtures containing 30 mM Tris-HCl, pH 7.1, 30 mM NH_4Cl, 1 mM 2 mercaptoethanol, 1.5% glycerol, 210 μg IF2 and 0.21 μg of trypsin (TPCK-treated) and when indicated, 1 mM GTP. Aliquots containing ~30 μg of IF2 were removed at the indicated times and the proteolysis was stopped by addition of 6 μg of bovine trypsin inhibitor and then analyzed on 15% SDS-containing PAGE. Electroblotting of peptides on PDVF membranes (Millipore) was carried out at 4°C for 1 h at 150 mA followed by 21 h at 350 mA as described [8]. Amino acid microsequencing was performed as previously described [9] in an Applied Biosystems pulse-liquid gas phase sequenator (model 477A).

3. RESULTS AND DISCUSSION

After checking that the presence of GTP does not affect the trypsinolysis of two non-GTP binding control proteins (not shown), we carried out an electrophoretic analysis of the time courses of trypsin digestion of IF2 with and without GTP (Fig. 1). The digestion patterns obtained were characteristic and reproducible and clearly different depending on the presence or absence of GTP during the digestion. Thus, in the absence of GTP, a characteristic high molecular weight product remains undigested for slightly longer time, while large differences are seen in the 40 kDa and 20-25 kDa region. Thus, a ca 40 kDa fragment remains intact in the presence of the ligand while other lower molecular weight fragments are completely absent or appear later in reduced amounts. This indicates that some peptide bonds become more and some less accessible to trypsin in the IF2-GTP complex (Fig. 1).

To localize the peptide bonds of IF2 which are more readily susceptible to trypsin hydrolysis and to identify those whose accessibility is influenced by GTP, the proteolytic fragments obtained under the various experimental conditions were separated electrophoretically, transferred by electroblotting onto membranes

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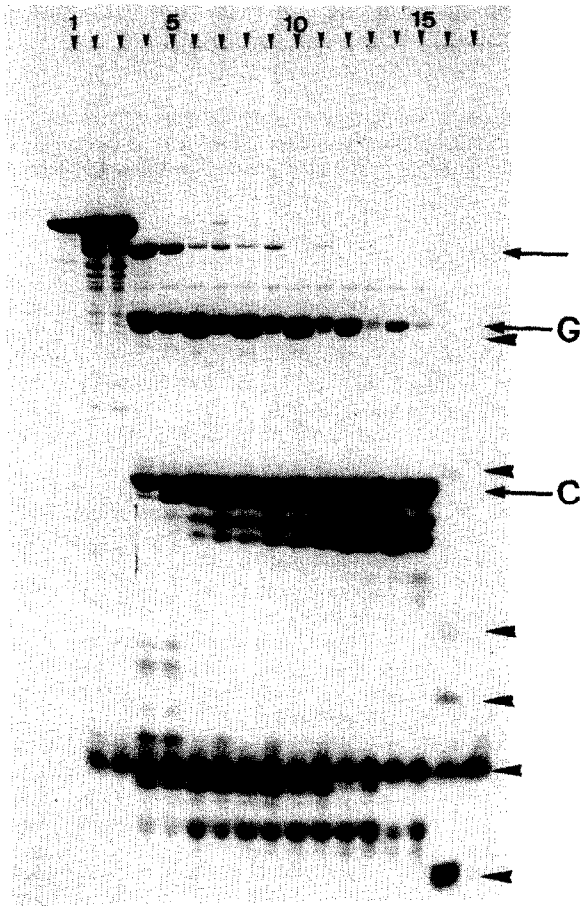

Fig. 1. Time course of trypsinization of *B. stearothermophilus* IF2 in the presence and absence of GTP. The experimental conditions are described in section 2. The lanes contained *B. stearothermophilus* IF2 digested with trypsin for the indicated time: (lane 1) undigested; (lanes 2,3) 0 min; (lanes 4,5) 5 min; (lanes 6,7) 15 min; (lanes 8,9) 20 min; (lanes 10,11) 30 min; (lanes 12,13) 60 min; (lanes 14,15) 120 min. The samples in the even-numbered lanes were incubated in the presence of GTP, those in the odd-numbered lanes in the absence of GTP. (Lane 16) Molecular weight markers indicated by arrowheads, ovalbumin (43 000), α-chymotrypsinogen (25 700), β-lactoglobulin (18 400), lysozyme (14 300), bovine trypsin inhibitor (6200), insulin (3000). (Lane 17) Bovine trypsin inhibitor. The ca 40 kDa G-fragment and the ca 24.5 kDa C-fragment are indicated by the arrows and the letters G and C, respectively. The uppermost arrow indicates the IF2 fragment remaining intact for longer time in the absence of GTP.

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                a          b          c
  1  MSKMRVYEYA KKQNVPSKDV IHKLKEMNIE VNNHMAMLEA DVVEKLDHQY
 51  RPKAEKKTET KNEKKAEKKT DKPKRPMPAK TADFSDEEIF DDVKEAAKPA
                                                    d
101  KKKGAAKGKE TKRTEAQQQE KKAFQAAKKK GKGPAKGKKQ AAPAAKQVPQ
                e
151  PAKKEKELPK KITFEGSLTV AELAKKLGRE PSEIIKKLFM LGVMATINQD
201  LDKDAIELIC SDYGVEVEEK VTIDETNFEA IEIADAPEDL VERPPVVTIM
                f
251  GHVDHGKTTL LDAIRHSKVT EQEAGGITQH IGAYQVTVND KKITFLDTPG
301  HEAFTTMRAR GAQVTDIVIL VVAADDGVMP QTVEAINHAK AANVPIIVAI
351  NKMDKPEANP DRVMQELMEY NLVPEEWGGD TIFCKLSAKT KEGLDHLLEM
401  ILLVSEMEEL KANPNRRAVG TVIEAKLDKG RGPVATLLVQ AGTLKVGDPI
451  VVGTTYGRVR AMVNDSGRRV KEAGPSMPVE ITGLHDVPQA GDRFMVFEDE
                g
501  KKARQIGEAR AQRQLQEQRS VKTRVSLDDL FEQIKQGEMK ELNLIVKADV
551  QGSVEALVAA LQKIDVEGVR VKIIHAAVGA ITESDISLAT ASNAIVIGFN
601  VRPDANAKRA AESEKVDIRL HRIIYNVIEE IEAAMKGMLD PEYEEKVIGQ
651  AEVRQTFKVS KVGTIAGCYV TDGKITRDSK VRLIRQGIVV YEGEIDSLKR
701  YKDDVREVAQ GYECGLTIKN FNDIKEGDVI EAYVMQEVAR A
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Fig. 2. Identification of the trypsin cleavage sites in the *B. stearothermophilus* IF2 molecule. The figure shows the complete primary structure of *B. stearothermophilus* IF2 [6]. The sequence of the blotted tryptic peptides determined by automated amino acid sequencing are indicated in bold letters and underlined. The individual cleavage sites identified are indicated by the lower case letters above the N-terminal amino acid of the corresponding peptide.

and subjected to automatic sequencing (see section 2). Comparing the N-terminal sequences obtained with the known primary structure of *B. stearothermophilus* IF2 [6] and taking into account the molecular weights of the fragments, we were able to identify all the main cleavage sites and to determine the approximate extension of the resulting peptides and their order of appearance (Figs 2 and 3). The sites affected by the GTP were also identified; these were the Arg-308–Ala-309 (Fig. 2, letter f) bond which is very efficiently protected in the IF2-GTP complex and the Lys-18–Asp-19 and Lys-25–Glu-26 bonds (Fig. 2, letters b and c) which are slightly less accessible in the free molecule than in the IF2-GTP complex. A schematic summary of the progress of trypsinolysis of IF2 is shown in Fig. 3: trypsin cuts in the order A, B, C and D (corresponding respectively to positions d, e, g and f of Fig. 2) while the N-terminal portion of IF2 (hatched in Fig. 3) is rapidly

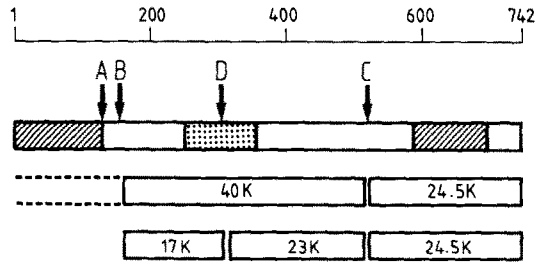

Fig. 3. Scheme indicating the main trypsin cleavage sites within the IF2 molecule, the sequential order of their appearance and the main digestion products. The first cleavages occur at A and B as well as at other identified (see Fig. 3) as well as unidentified sites within the N-terminal portion of the molecule (broken bar) which is rapidly degraded to small products. The next cleavage occurs at C to yield a 24.5 kDa C-fragment and a 40 kDa G-fragment. In the absence of ligands, the latter is cleaved at position D to yield the 17 kDa and 23 kDa fragments which are eventually degraded further. The dotted area indicates the GTP-binding domain. The two striped areas indicate the regions of the IF2 molecule that have been deleted by manipulation of the *infB* gene (see text).

degraded yielding small and discrete peptides of $M_w \leqq 5000$. It is noteworthy that this N-terminal region of the molecule upstream of the A cleavage site displays a very low degree of conservation among the four IF2 molecules whose sequences are known sofar [2,10] and that removal of this portion of the molecule by proteolysis [7] or by genetic deletion [10] yields a fragment substantially active in all translational functions of IF2 tested in vitro.

The cleavage at the C site produces a ~24–25 kDa fragment corresponding to the entire (or virtually entire) C-terminal fragment of the protein; this represents a structurally compact domain which resists even long digestion periods with trypsin or with other proteolytic enzymes (not shown). An internal deletion of the *infB* gene removing the central portion of this domain (indicated by the hatched area in Fig. 3) was found to abolish completely the IF2 functions in protein synthesis (not shown).

The cleavage site indicated with D is located in the center of the four structural elements constituting the guanosine nucleotide binding site of IF2 (indicated by the dotted area) which are identical to those of EF-Tu [11] and highly conserved among all known G-proteins [12]. In the absence of GTP, cleavage at the D site occurs readily yielding two discrete fragments (17 kDa and 23 kDa) which, though relatively resistant to proteolysis, are eventually degraded to smaller products. In the presence of GTP, however, the D site becomes inaccessible and a 40 kDa fragment remains intact even after a long incubation with trypsin or chymotrypsin (not shown).

The present results indicate the existence of two fairly compact domains in the IF2 molecule, the central (G-domain) and the C-terminal domain (C-domain). These domains are separate entities not only structurally but also functionally, since the G-domain carries the ribosomal and the GTP binding sites of the factor while the C-domain contains the fMet-tRNA binding site ([13] and manuscript in preparation).

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