Limited Proteolysis of Yeast Elongation Factor 3

SEQUENCE AND LOCATION OF THE SUBDOMAINS*

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Elongation factor 3 (EF-3) is an ATPase essential for polypeptide chain synthesis in a variety of yeasts and fungi. We used limited proteolysis to study the organization of the subdomains of EF-3. Trypsinolysis of EF-3 at 30 °C resulted in the formation of three fragments with estimated molecular masses of 90, 70, and 50 kDa. Yeast ribosomes protected EF-3 and the large fragments from further degradation. ATP exposed a new tryptic cleavage site and stabilized the 70- and 50-kDa fragments. The conformation of EF-3 as measured by fluorescence spectroscopy did not change upon ATP binding. Poly(G) stimulated proteolysis and quenched the intrinsic fluorescence of EF-3. Using gel mobility shift, we demonstrated a direct interaction between EF-3 and tRNA. Neither tRNA nor rRNA altered the tryptic cleavage pattern. The proteolytic products were sequenced by mass spectrometric analysis. EF-3 is blocked NH2-terminally by an acetylated serine. The 90-, 70-, and 50-kDa fragments are also blocked NH2-terminally, confirming their origin. The 50-kDa fragment (Ser2-Lys443) is the most stable domain in EF-3 with no known function. The 70-kDa fragment (Ser2-Lys668) containing the first nucleotide-binding sequence motif forms the core ATP binding subdomain within the 90-kDa domain. The primary ribosome binding site is located near the loosely structured carboxyl-terminal end.

Translational elongation in fungi requires a third protein, elongation factor 3 (EF-3), in addition to elongation factors 1 and 2 (1–3). EF-3 is an ATPase. The intrinsic ATPase activity (80 S-independent) of EF-3 is stimulated 2 orders of magnitude by EF-3-dependent functions (12). In the present paper, we report the effects of ligands on the proteolytic cleavage and on the domain structure of EF-3.

MATERIALS AND METHODS

All materials used were obtained from standard sources as described in previous papers (12, 15). Yeast 80S ribosomes and the subunits were prepared essentially as described (15). EF-3 was purified to homogeneity from an overexpressing yeast strain containing the plasmid-borne YEF3 gene according to Kambampati and Chakraburtty (16). SDS-polyacrylamide gel electrophoresis was performed using 10% gels as described (17). For Western blotting, proteins were transferred electrophoretically to polyvinylidene difluoride membrane and developed with a polyclonal antibody to EF-3 (18). [35S]Protein A was used as a secondary antibody for the detection of anti-EF-3 cross-reacting proteins.

ATPase Activity Assays—The nucleotide hydrolytic activity of EF-3 was measured under standard assay conditions (15). To measure the ribosome-stimulated ATPase activity of EF-3, 6.3 pmol of twice washed yeast ribosomes were included in the reaction. The amount of ATP released from [γ-32P]ATP was measured according to our previously published protocol (15).

Tryptic Digestion of EF-3—EF-3 (0.6 mg/ml) in 0.1 M Tris/HCl, pH 8.0, was digested at 30 °C with TPCK-trypsin at an EF-3/trypsin (w/w) ratio of 125:1. Reactions were carried out in buffer A (25 mM Tris/HCl, pH 7.5, 10 mM Mg(OAc)2, 50 mM NH4Cl, 1 mM diithiothreitol, 3% glycerol). Aliquots containing 2 μg of EF-3 were removed at various times, and the digestions were terminated with a 5-fold excess (w/v) of soybean trypsin inhibitor. Samples were either assayed for the ATPase activity or diluted into SDS sample buffer, heated at 90 °C for 10 min, and subjected to electrophoresis.

Fluorescence Measurements—All measurements were carried out at room temperature (22 °C) using a SLM 4800C spectrofluorometer equipped with xenon arc light source. Slits of 4-nm width were used for excitation and emission. The emission spectra of EF-3 (10 μg/ml in buffer A) were recorded by excitation at 294 nm in a rectangular quartz cuvette (inner diameter, 10 mm). The observed fluorescence intensities were corrected by subtracting the appropriate blanks and are expressed in arbitrary units.

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* The abbreviations used are: EF, elongation factor; NBS, nucleotide binding sequence; TPCK, tosylphenylalanyl chloromethyl ketone; HPLC, high performance liquid chromatography.

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FIG. 1. Time course of tryptic digestion of EF-3. EF-3 was digested at 30 °C with TPCK-trypsin as described under "Materials and Methods." The digests were electrophoresed on 10% SDS-polyacrylamide gel and stained with Coomassie Blue. Lanes 1–8 represent 2 μg of EF-3 tryptic digests at 0, 1, 2, 5, 10, 20, 30, and 60 min, respectively. The standard protein markers (lane MW) are indicated on the right. The positions of EF-3 and the tryptic peptides are indicated on the left.

**A**

**B**

FIG. 2. ATPase activity of EF-3 during the time course of tryptic digestion. EF-3 was digested with trypsin at 30 °C as described under "Materials and Methods." Aliquots taken at different time intervals were assayed for the ATPase activity in the absence (panel A) or presence (panel B) of yeast ribosomes.

Tryptic cleavage destroyed both the intrinsic and 80S-stimulated ATPase activity of EF-3 (Fig. 2). However, the inactivation kinetics were different for these two functions (see insets in Fig. 2). The ribosome-stimulated ATPase activity was destroyed quantitatively within 10 min of the reaction (Fig. 2B). The digested proteins retained about 10% of the intrinsic ATPase activity (Fig. 2A). Neither the intact EF-3 nor fragment A was visible at this time point (Fig. 1, lane 5). We attribute the 10% residual intrinsic ATPase activity to fragment B2. Further studies with the isolated peptides will be necessary to confirm the hydrolytic activity of the truncated fragment. The loss of more than 90% of the ribosome-stimulated function of EF-3 within the first few minutes of tryptic digestion (Fig. 2B) strongly suggests that the initial cleavage that generated fragments A and B2 destroyed the primary ribosome binding site of the protein. This conclusion is in agreement with our previously published data (12).

In an attempt to analyze the effects of substrate binding on the domain structure of EF-3, tryptic digestions were carried out in the presence of 80S ribosomes, ATP, polynucleotides, rRNA, and tRNA (Figs. 3–6). Data presented in Fig. 3 show the tryptic digestion of EF-3 in the presence and absence of ribosomes. These experiments were conducted exactly as reported in our previous publication (12) except in the present studies, the reactions were at 30 °C instead of 0 °C. The tryptic fragments of EF-3 were visualized by Western blotting to avoid interference by the ribosomal proteins. A comparative analysis of the immunoblot data presented in Fig. 3 with the Coomassie-stained gel in Fig. 1 revealed some interesting differences. First of all, EF-3 was relatively more resistant to tryptic digestion in the presence of the ribosome even with 10-fold excess of enzyme (the ratio of EF-3 to trypsin was 12.5:1 instead of 125:1). This amount of trypsin was shown to have minimal effect on the structure and the conformation of the ribosome (12, 21). Peptides B1 and C1 were also protected from tryptic digestion as evidenced by the accumulation of these two fragments in the presence of the ribosome (Fig. 3), the protective effect of the ribosome on the carboxyl-terminally derived C1 peptide was evidenced by the accumulation of these two fragments in the presence of the ribosome (Fig. 3), the protective effect of the ribosome on the carboxyl-terminally derived C1 peptide was evidenced by the accumulation of these two fragments in the presence of the ribosome (Fig. 3), the protective effect of the ribosome on the carboxyl-terminally derived C1 peptide was evidenced by the accumulation of these two fragments in the presence of the ribosome (Fig. 3), the protective effect of the ribosome on the carboxyl-terminally derived C1 peptide was evidenced by the accumulation of these two fragments in the presence of the ribosome (Fig. 3). The relative stability of the fragments B1 and C from trypsinolysis suggests the possible existence of two distinct subdomain structures within fragment A. The low molecular weight peptides (<30 kDa) that appeared at 1 min (lane 2) were completely degraded by 10 min of digestion (lane 5). A similar proteolytic cleavage pattern for EF-3 was reported by Miyazaki et al. (13) and Ladror et al. (14). Data reported by Miyazaki et al. showed the presence of an additional proteolytic fragment B2, which we observed only when EF-3 was digested in the presence of ATP (see Fig. 4A).
EF-3 interacts with ATP, tRNA, rRNA, and various synthetic oligonucleotides (15, 16, 20, 23–25). Data presented in Fig. 4A demonstrate the effect of ATP on the trypsinolysis and on the intrinsic fluorescence of EF-3. Comparative analysis of the rate of proteolysis of EF-3 in the absence and presence of ATP clearly demonstrates the protective effect of ATP on EF-3. The nucleotide also protected the A and B1 peptides. A doublet band, designated as B2 peptide, appeared with an electrophoretic mobility similar to that of B1 peptide (Fig. 4A, lane 3). The 90-kDa A peptide was generated by cleavage of the most trypsin-sensitive bond in EF-3 within NBSII (12). This cleavage site was protected by ATP. A new trypsin-sensitive bond in NBSI was exposed in the presence of ATP, which resulted in the formation of fragment B2 (for the amino acid sequence, see Fig. 7). Similar results were reported by Miyazaki et al. (13) for EF-3 and by Yike et al. (22) for the cystic fibrosis transmembrane conductance regulator protein CFTR. These results strongly suggest a change in the conformation of EF-3 upon ATP binding. However, there was no significant change in the intrinsic fluorescence of the protein in the presence of ATP (Fig. 4B). We conclude that ATP changed the conformation of EF-3 around its binding sites without causing a global change in the overall conformation of the protein. ATP showed no protective effect on the carboxyl-terminally derived C1, C2, and C3 peptides. It will be of interest to investigate the role of ATP in the activation of the ribosome-stimulated hydrolitic function of EF-3 similar to that observed for the GTP-binding proteins EF-1α (EF-Tu) and EF-2 (EF-G) (26).

The functional dependence of the yeast ribosomes on EF-3 strongly suggests that the protein may interact directly with rRNA. In the present studies, we investigated the effect of synthetic oligonucleotides and ribosomal RNA on the proteolytic cleavage pattern and on the intrinsic fluorescence of EF-3. Of the three homopolynucleotides tested, only poly(G) showed the most significant effect. The effect was visible when the trypsinolysis was carried out at 30 °C and also at 0 °C (Fig. 5, A and B). In the presence of poly(G), no detectable amount of the full-length EF-3 or fragment A was visible at the end of the 10-min digestion time (Fig. 5A). The cleavage pattern in the absence of oligonucleotides was very similar to those with poly(U) and poly(C). Fragment C (50 kDa) was formed only when EF-3 was digested at 30 °C (Fig. 5A, EF-3 alone). However, in the presence of poly(G), fragment C was formed even when the reactions were carried out at 0 °C (Fig. 5B, + poly(G)). The binding studies as measured by fluorescence quenching (Fig. 5C, filled circles) reflected a similar effect of poly(G). We reported previously a high affinity binding of EF-3 to a guanosine-rich sequence in RNA (23, 24). An altered proteolytic cleavage pattern and fluorescence quenching by poly(G) suggest a possible conformation change of the protein upon binding to a guanosine-rich sequence in rRNA. However, neither 18S nor 26S rRNA separately or as a mixture showed any detectable effect on the proteolysis of EF-3 (data not shown).

The primary function of EF-3 is to remove deacylated tRNA from the ribosomal exit site (E-site) and stimulate binding of the ternary complex to the A-site (6, 25). The deduced amino acid sequence of EF-3 revealed the presence of a putative aminoacyl-tRNA synthetase sequence motif (10). Based on this information, we examined for a possible interaction between EF-3 and tRNA by gel mobility shift assays. Data presented in Fig. 6A demonstrate that indeed, the mobility of tRNA was retarded upon interaction with EF-3 (lanes 2–6). A nonspecific protein, bovine serum albumin, had no effect (lane 7). It should be noted that the complex was not detected when electrophoresis was continued for longer than 10–15 min. The disappearance of the EF-3-tRNA complex does not represent degradation of either tRNA or the protein because these macromolecules remained intact at the end of the experiment (data not shown). However, tRNA did not change the trypptic cleavage pattern of the protein (Fig. 6C). The labile nature of the EF-3-tRNA complex and the inability of tRNA to protect it from proteolysis may be a reflection of a weak interaction between these two macromolecules.
To analyze further the domain structure of EF-3, several of the tryptic fragments were sequenced by mass spectrometry. Coomassie-stained bands from the gel (Fig. 1) were excised and subjected to complete tryptic digestion. The peptides were sequenced using a mass spectrometer as described under “Materials and Methods.” The peptides identified in each fragment are listed in Tables I–III.

As noted in the earlier section, trypsinolysis of EF-3 at 0 °C resulted in the cleavage of a single peptide bond generating a large 90-kDa fragment (N peptide) and a small 30-kDa fragment (C1 peptide). The N peptide is equivalent to fragment A described in the current studies (Fig. 1). The C1 peptide originated from the carboxyl-terminal end and contains the amino acid residues Gln775-Phe1044 (12). Attempts to sequence fragments A, B1, and C were unsuccessful because all three were amino-terminally blocked. We conclude that these fragments are from the NH2-terminal end. The 90-kDa fragment contains residues Ser2-Arg774. This domain of EF-3 was stable to further proteolysis at 0 °C and retained the intrinsic ATPase activity (12). Our results indicate that proteolysis at 30 °C exposed additional trypsin-sensitive bonds in the 90-kDa domain, resulting in the formation of a stable 50-kDa fragment through the intermediate formation of the 70-kDa fragment (Fig. 1).

Data presented in Table I show the bulk of the peptides identified in the 70-kDa fragment. Tandem mass spectrometry sequencing confirmed the presence of an NH2-terminally blocked octapeptide (2SDSQKSIR) containing an acetylated serine. Analysis of the total tryptic digest of the 70-kDa fragment identified the presence of the peptide Phe650-Lys660, but peptides Val669-Arg676 and Ile697-Lys707 were absent. Peptides Thr661-Lys666 and Gln663-Lys664 were too small for the detection by LCQ mass spectrometric analysis. We failed to identify the peptide Ala665-Lys668 (430 Da) in the total digest of the full-length EF-3 and also in the 70-kDa fragment. This peptide may or may not be present in the 70-kDa fragment. Based on these analyses, we concluded that the 70-kDa fragment of EF-3...
Domain Organization of EF-3

Fig. 7. Amino acid sequence of EF-3 and the locations of the tryptic fragments. The purine nucleotide binding consensus sequences are single underlined, and the phosphate binding sequences are double underlined. The polylysine residues at the carboxyl-terminal end are protected by the ribosome. Fragment begins at residue 2 and ended somewhere between the residues 660 and 685 (Fig. 7). The 70-kDa fragment contains one complete nucleotide-binding cassette (NBSI). ATP protected this fragment from further degradation (Fig. 4). We hypothesize that the 70-kDa fragment forms the core ATP binding subdomain within the NH2-terminal derived 90-kDa domain.

The 70-kDa fragment gave a mass of 48.4 kDa in the LCQ mass spectrometer (data not shown). The last peptide confirmed in the total tryptic digest of the 50 kDa fragment was Ala511-Lys483. Peptide Ile444-Lys438 and those COOH-terminal to this peptide were absent in the 50-kDa fragment. Thus, the 50-kDa fragment spans from Ser2 to Lys443. The calculated molecular mass of 48.38 kDa is in good agreement with the size of this fragment.

This paper and elsewhere (12, 14) strongly suggest the existence of at least two organized functional domains in EF-3. The protein also contains two trypsin-resistant cores (B1 and C) near the NH2-terminal end. Our results demonstrate that the lysine and arginine residues in NBSI (residues 463–575) are relatively inaccessible to trypsin compared with those in NBSII (residues 701–928). The most trypsin-sensitive bond of EF-3 (Arg274-Gln775) is located within NBSII (between the purine binding sequence and the phosphate binding loop, see Fig. 7). The sequence around this cleavage site is exposed and may function as a hinge between the stable amino-terminal domain and the flexible carboxyl-terminal domain. The phosphate binding loop of the second nucleotide binding domain along with the putative aminocyl-tRNA synthetase homology region (residues 820–865) and the positively charged polylysine blocks (residues 1009–1031) form a loosely structured carboxyl-terminal domain that contains the primary ribosome binding site of the protein (12). The protective effect of yeast ribosomes on the trypsinoysis of EF-3 suggests that the cleavage sites Arg542, Arg970, and the lysine residues located near the carboxyl-terminal end are protected by the ribosome. Fragment C (residues 2–443) is extremely resistant to trypsin (12, 14). The functional significance of this stable domain near the NH2-terminal end of EF-3 remains undefined. Yeast strains expressing truncated forms of EF-3 (lacking Glu12-Ala411) were nonviable (27).

Further work with the purified EF-3 fragments is needed to fully understand the structural organization of EF-3. Attempts to crystallize the full-length protein were unsuccessful (Kambampati & Chakraburtty, unpublished data). The loosely structured carboxyl-terminal end may have interfered with the crystal formation. With the identification of several functional subdomains, it should be feasible to determine the structures of the individual domains by x-ray crystallography.

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