Domain structure analysis of elongation factor-3 from *Saccharomyces cerevisiae* by limited proteolysis and differential scanning calorimetry

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(RECEIVED December 13, 1997; ACCEPTED August 20, 1998)

Abstract

Elongation-factor-3 (EF-3) is an essential factor of the fungal protein synthesis machinery. In this communication the structure of EF-3 from *Saccharomyces cerevisiae* is characterized by differential scanning calorimetry (DSC), ultracentrifugation, and limited tryptic digestion. DSC shows a major transition at a relatively low temperature of 39 °C, and a minor transition at 58 °C. Ultracentrifugation shows that EF-3 is a monomer; thus, these transitions could not reflect the unfolding or dissociation of a multimeric structure. EF-3 forms small aggregates, however, when incubated at room temperature for an extended period of time. Limited proteolysis of EF-3 with trypsin produced the first cleavage at the N-side of Gln775, generating a 90-kDa N-terminal fragment and a 33-kDa C-terminal fragment. The N-terminal fragment slowly undergoes further digestion generating two major bands, one at ~75 kDa and the other at ~55 kDa. The latter was unusually resistant to further tryptic digestion. The 33-kDa C-terminal fragment was highly sensitive to tryptic digestion. A 30-min tryptic digest showed that the N-terminal 60% of EF-3 was relatively inaccessible to trypsin, whereas the C-terminal 40% was readily digested. These results suggest a tight structure of the N-terminus, which may give rise to the 58 °C transition, and a loose structure of the C-terminus, giving rise to the 39 °C transition. Three potentially functional domains of the protein were relatively resistant to proteolysis: the supposed S5-homologous domain (Lys102–Ile368), the N-terminal ATP-binding cassette (Gly463–Lys622), and the aminoacyl-tRNA-synthase homologous domain (Glu820–Gly865). Both the basal and ribosome-stimulated ATPase activities were inactivated by trypsin, but the ribosome-stimulated activity was inactivated faster.

Keywords: elongation factor 3, EF-3; differential scanning calorimetry; limited proteolysis; *Saccharomyces cerevisiae*

Yeast elongation factor 3 (EF-3) is an essential requirement for the protein translation machinery that is unique to fungi (Skogerson & Wakatama, 1976; Qin et al., 1990; Sandbaken et al., 1990; Belfield et al., 1995). A single gene, YEF-3, encodes the protein of 1,044 amino acids with calculated average molecular weight of ~116,000 (Qin et al., 1990; Sandbaken et al., 1990). The biological role of EF-3 is not completely understood. However, the protein is necessary for two functions of the ribosome; the binding of aminoacyl tRNA to the ribosomal aminoacyl site (A site) when the peptidyl and exit sites (P and E sites) are occupied; and for dissociation of bound tRNAs from the ribosomal E site (Kamath & Chakraburty, 1989; Triana-Alonso et al., 1995). Both these processes are dependent on ATP hydrolysis by EF-3 (Triana-Alonso et al., 1995).

These results confirmed earlier observations showing that EF-3 was necessary for EF-1 α -dependent binding of aa-tRNA to the ribosome (Uritani & Miyazaki, 1988a; Kamath & Chakraburty, 1989). EF-3 possesses ATPase activity that is stimulated up to a 100-fold by yeast ribosomes (Uritani & Miyazaki, 1988b; Kamath & Chakraburty, 1989), and is required for in vitro protein synthesis.

Sequence and homology analyses suggest that the single polypeptide chain of EF-3 consists of several functional domains. The amino terminal domain may be homologous to the eubacterial ribosomal protein S5 and may function in rRNA binding and translational accuracy (Belfield et al., 1995). The protein has an internal repeat of about 100 residues, each of the repeats containing an ATP binding cassette (ABC) (Qin et al., 1990; Sandbaken et al., 1990; Belfield et al., 1995). Toward the C-terminus is an aminoacyl tRNA synthetase-homologous region that may function in tRNA association (Belfield et al., 1995). Finally, the C-terminal 56 residues embody a highly charged region with 33 charged residues.

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This region probably functions in ribosome binding, as indicated by the following: (1) a monoclonal antibody to this region inhibited the ribosome-dependent ATPase activity but not the basal ATPase (Uritani et al., 1996); and (2) poly-(Asp) and poly-(Glu) polyamino acids also inhibited the ribosome-dependent activity but not the basal ATPase activity (Uritani et al., 1994). A concurrent study (Kambampati & Chakraburty, 1997) has recently shown that ribosome-binding domain resides in the C-terminal 30-kDa fragment of EF-3.

The large size of EF-3 is expected to hinder the structural determination of the full-length protein. Therefore, we are interested in dissecting EF-3 into functional or structural domains, which are more amenable to structural determination by NMR or X-ray crystallography techniques. For that goal, we characterize the thermal and hydrodynamic behavior of full length EF-3, and perform limited tryptic digestion to determine the domain accessibility and assign functional activities to specific structural domains.

Results

Differential scanning calorimetry and heat inactivation of EF-3

DSC studies of large multidomain proteins like EF-3 can reveal the thermodynamic parameters of the unfolding of each of the protein domains. Two thermal transitions were observed when EF-3 was scanned from 10 to 80°C: a sharp peak with large heat capacity at about 39°C and another smaller peak at about 58°C (Fig. 1). The 39°C peak indicates that EF-3 alone may be significantly unfolded at physiological temperatures (e.g., 37°C); however, the presence of ribosomes and other proteins may prevent EF3 from unfolding. Furthermore, the sharpness of the 39°C peak suggests a cooperative unfolding event, where the unfolding of one structure or domain triggers the unfolding of other related structures; the released heat is the total from all the unfolding structures. The second peak is small and represents a more stable domain. A more rigorous analysis of the unfolding at 39°C would require calculating van't Hoff and calorimetric enthalpies (Sanchez-Ruiz, 1995). However, this analysis was precluded by two factors observed in these ex-

Fig. 1. Differential scanning calorimetry of EF3. EF3 at 81 mM (1.5 mL of 9.4 mg/mL solution) was scanned at a rate of 30 or 60°C/h, in 50 mM NaHPO₄, pH 7.5, 10 mM MgCl₂, and 1 mM DTT.

periments: (1) aggregation of the protein was observed during the 39°C unfolding event, which indicates that the unfolding is irreversible (S. Snyder, unpubl. results); and (2) the transition temperatures were dependent on the DSC scan rate (Fig. 1). For this analysis, the condition of scan-rate independence must be met if the transition is irreversible (Sanchez-Ruiz et al., 1988).

The DSC results suggest that EF-3 is likely to be heat sensitive. Indeed, even a short exposure of 1 min to 45°C inactivated the ribosome-stimulated ATPase activity by about 85% (Fig. 2). A 5-min exposure to 45°C completely inactivated the ATPase activity. However, in the presence of ribosomes, a 1-min exposure to 45°C inactivated the ATPase by only 60% (Fig. 2). While incomplete, the protection against heat inactivation of EF-3 by ribosomes suggests that ribosomes may stabilize the EF-3 also at physiological temperatures.

Analytical ultracentrifugation

Hydrodynamic measurements of EF-3 were carried out by sedimentation equilibrium and sedimentation velocity, and the data were analyzed as described previously (Holzman & Snyder, 1994; Snyder et al., 1996). Equilibrium experiments that were performed in the absence of glycerol showed one sedimenting component with a molecular mass of 116 kDa, which is in a good agreement with the calculated molecular mass of 115,977 Da (Fig. 3A). This result appears to be in accord with Dasmahapatra and Chakraburty (1981); however, these authors did not specify the experimental conditions (Fig. 3 in Dasmahapatra & Chakraburty, 1981). The equilibrium experiments were repeated at 20°C in the presence of 10% glycerol to more closely simulate the condition of other experiments. These runs showed that 60% of the protein was in aggregated form with a mass of about 380 kDa (S. Snyder, unpubl. results). In the presence of glycerol, 48 h were required for the samples to reach hydrodynamic equilibrium. Therefore, aggregation could have occurred during the time course of the experiment. To resolve this point, a sedimentation velocity experiment was

Fig. 2. Effect of incubation of at 45°C on ribosome-stimulated ATPase activity of EF-3. EF-3 alone (filled triangles), ribosomes alone (filled squares), or EF-3 together with ribosomes (open circles) were incubated at 45°C, aliquots were taken out at 1, 2, and 5 min, and ATPase was assayed in the presence of ribosomes by the malachite green procedure, as indicated in Materials and methods.

Fig. 3. Analytical ultracentrifugation of EF-3. **A:** Sedimentation equilibrium run of EF-3 for 64 h at 12,000 rpm, in 60 mM Tris, 20 mM NH_4Cl , 5 mM MgCl_2 , 2 mM DTT, pH 7.8, and 4 °C, analyzed as described by Snyder et al. (1996). **B:** Sedimentation velocity of EF-3 following a 92-min spin at 42,000 rpm, in 10% glycerol, and 30 mM Tris pH 8.0, at 20 °C after 48-h incubation at -80°C . The sedimentation coefficients were corrected for standard conditions in water at 20 °C. **C:** Sedimentation velocity as in **B** after a 48-h incubation at room temperature. **D:** Analysis of sedimentation coefficient distributions (Stafford, 1992) of the velocity runs shown in **B** and **C**.

performed with samples that were stored for 48 h at either -80°C or room temperature (Fig. 3B,C). This experiment showed that the sample from -80°C is monomeric, with an average sedimentation coefficient of 5.4, which is consistent with an MW of about 110 kDa (Fig. 3B). Conversely, the sample stored at room temperature had a large proportion of the protein in aggregated form (Fig. 3C). An analysis of sedimentation coefficient distributions of these data (Stafford, 1992) confirmed that the proportion of protein sedimenting with a coefficient of ~ 5 S was considerably less in the sample incubated at room temperature, while the proportion of larger material was increased relative to the sample incubated in the freezer (Fig. 3D). These results indicate that freshly thawed material was monomeric. However, EF-3 had a tendency to aggregate at room temperature to form trimers or tetramers as well as larger aggregates.

SDS PAGE of trypsin-treated EF-3

Limited tryptic digestion of EF3 has been reported previously in the context of demonstrating protection of EF-3 by nucleotides (Miyazaki et al., 1988) or by ribosomes (Kambampati & Chakraburty, 1997). In these reports, however, no attempts were made to identify various cleavage sites, and no structural information was derived. In this study, the first cleavage observed after a ~ 10 -s trypsin proteolysis of EF-3 generated two major bands, one

of about 90 kDa, and the other of 33 kDa (Fig. 4A, lane 0.2). The 33-kDa band was quickly digested further, and four bands with molecular weights of 32, 31, 29, and 28 kDa appeared. The 32- and 31-kDa bands were blotted on PVDF and sequenced, and both gave the EF-3 sequence starting at position Gln775. Another band of 13 kDa that appeared after longer incubation times also gave the sequence starting at position 775. Cleavage at position 775 should generate two fragments with calculated MWs of 85,537 and 30,457 Da, respectively. The full length EF-3 protein with calculated average MW of 115,977 Da, migrated as a 125-kDa band (Dasmahapatra & Chakraburty, 1981; Miyazaki et al., 1988; Uritani & Miyazaki, 1988a; this work). Therefore, the 85- and 30-kDa fragments most likely correspond to the 90- and 33-kDa bands, respectively. Thus, we conclude that cleavage at Gln775 is the first observable cleavage to occur. This conclusion is in accord with Kambampati and Chakraburty's suggestion (1997), but they identified only the N-terminal Gln of the 30-kDa fragment, and therefore could not reliably distinguish between a cleavage at position Gln775 and Gln738.

The 90-kDa fragment was slowly digested further to form two major bands with an apparent MW of 55 and 75 kDa, respectively. The 55-kDa fragment was unusually resistant to trypsin digestion; thus, even a 5-h incubation, which completely digested all the other visible bands, did not eliminate this fragment (Fig. 4B).

Fig. 4. SDS-PAGE of limited digestion of EF-3 by trypsin. Incubation times are shown above the lanes in minutes. "S" denotes molecular weight standards from 10 to 120 kDa in increments of 10 kDa. EF-3 was dialyzed into 25% glycerol and 30 mM Tris pH 8.0 and adjusted to 1.2 mg/mL. Trypsin was added at a protein to trypsin ratio of 50:1. The reactions were stopped by transferring an aliquot to a tube containing the SDS-PAGE sample buffer.

Repeated attempts to sequence the 55- and 75-kDa fragments gave no results. However, acid digestion of the 55-kDa fragment produced sequences starting at Pro304, Pro280, and Glu207. Sequencing of the full-length EF-3 also failed, probably because the amino terminus was blocked. Taken together, these data suggest that both the 55- and 75-kDa fragments contain the blocked amino terminus.

Sequence analysis of EF-3 shows that cleavage at Arg474 would generate a fragment of 51,971 Da. A tryptic fragment of Ala475–Arg491 was detected by HPLC after a 30-min proteolysis (see below). Because the full-length protein migrates on SDS-PAGE as a larger protein, we expect this cleavage to generate the 55-kDa band. Similarly cleavages at Lys649 or Lys668 would generate fragments of 71,470 and 73,585 Da, respectively. Both fragments starting at Val669 and Phe650 were found by HPLC (see below); therefore, either or both of these cleavages may have generated the 75-kDa band.

RP HPLC of partially digested EF-3

A chromatogram of a 30-min tryptic digest of EF-3 is shown in Figure 5A. Peptides were collected and analyzed by sequencing and mass spectrometry. Figure 5B shows the observed cleavage

sites in relation to EF-3 structure and its putative functional regions according to Belfield et al. (1995). Except for the first 56 residues, the N-terminal 60% of EF-3 was relatively inaccessible to trypsin, indicating a tight structure. Apparently, these cleavages were frequent enough to be detected by RP-HPLC, but not by SDS-PAGE (Figs. 5 and 4, respectively). In contrast, the C-terminal 40% was readily digested by trypsin, as was reported by Kambampati and Chakraborty (1997). Our analysis reveals three potentially functional domains of the protein that were inaccessible to trypsin. In the N-terminal half of EF-3, the putative S5-homologous domain from Lys102 to Ile368 (Belfield et al., 1995) and the N-terminal ATP-binding cassette from Gly463 to Lys622 were resistant to digestion. In addition, the aminoacyl-tRNA-synthase homologous domain, which is located in the trypsin-susceptible C-terminal half from Glu820 to Gly865, was also resistant to trypsin digestion (Fig. 5B).

Fig. 5. HPLC chromatogram of a 30-min tryptic digest of EF-3 at 22°C (A), and the peptides observed after a 30-min tryptic digest (B). Bold characters indicate beginning of an identified peptide; gray areas indicate identified peptides; boxes indicate functional regions according to (Belfield et al., 1995). Tryptic peptides were separated by reversed phase HPLC and analyzed by Edman sequencing and by mass spectrometry.

Effect of partial tryptic digestion on ATPase activity of EF-3

A sample of EF-3 was digested as described above with a protein to trypsin ratio of 50:1 (Fig. 6) or 125:1 (not shown). After incubation, aliquots were transferred to a tube containing soybean trypsin inhibitor (STI) at a weight ratio of STI to trypsin of 4:1 and placed on ice until assayed for ATPase activity. These experiments show that both basal and ribosome-stimulated ATPase activities were inactivated by tryptic cleavage; however, the ribosome-stimulated activity was inactivated faster (Fig. 6). The rate of loss of these activities was fit to one or two component models using Origin™ software. Modeling the rate of loss of basal ATPase activity with a single component model gave $\chi^2 = 6.8$ and with a two-component model $\chi^2 = 0.9$. Similarly, modeling the rate of loss of ribosome-stimulated activity with a single component gave $\chi^2 = 25.5$, and with a two-component model $\chi^2 = 8.3$. Thus, the loss of ATPase activities fits a second-order rate rather than the expected

pseudo-first-order for both the basal and ribosome-stimulated activities.

Discussion

The yeast EF-3 is a multidomain protein. It comprises several domains that may bind various forms of RNA (Belfield et al., 1995) and two domains that contain an ATP-binding cassette (Qin et al., 1990; Sandbaken et al., 1990; Belfield et al., 1995). We attempted to obtain additional information of the domain structure by differential scanning calorimetry (Fig. 1). The DSC experiments confirmed the multidomain structure of EF-3 where a part of the protein unfolded cooperatively at a relatively low temperature of 39 °C, and another part formed a relatively stable structure that unfolded at a higher temperature of 58 °C. The relatively low transition temperature indicates that a part of the protein unfolds readily. Such a domain might be easily accessible to proteases, as was observed with the C-terminal 30-kDa fragment of the protein. Unfortunately, a more rigorous analysis of the thermal behavior was not possible because the unfolding was irreversible and scan rate dependent. The DSC profile of EF-3 does not reflect the dissociation of a multimeric structure, as indicated by the ultracentrifugation experiments, which show that fresh EF3 preparations were monomeric.

The quaternary structure of EF-3 was analyzed by analytical ultracentrifugation under conditions similar to those of the DSC or tryptic digestion experiments. EF-3 was found to be a monomer when analyzed immediately after thawing. However, the protein formed small aggregates if left at room temperature for about 48 h. This aggregation was not associated with loss of activity as the protein retained almost 90% of its activity during such incubation (J. Capobianco, unpubl. results). Therefore, the aggregation that we observe is not likely to hinder *in vitro* protein synthesis. These data also indicate that tryptic digestion of EF-3 does not reflect inaccessibility of quaternary structure of the protein.

Kambampati and Chakraburty (1997) reported that the first tryptic cleavage in EF-3 was at the N-terminal side of Gln775; however, they sequenced only the first Gln of the C-terminal 30-kDa fragment, and therefore, could not distinguish between cleavages at positions Gln738 and Gln775. Our results confirm that the first cleavage is at position 775. This cleavage would be expected to prevent ribosome-dependent ATPase activity, as it separates between the two ATP-binding domains and the ribosome binding site, which resides in the C-terminal 30-kDa fragment of EF-3 (Qin et al., 1990; Uritani et al., 1994, 1996; Belfield et al., 1995; Kambampati & Chakraburty, 1997). In addition, this cleavage may inhibit some of the basal ATPase activity because it would destroy the C-terminal ATP-binding cassette, which extends from Gly701 to Asp929 (Qin et al., 1990; Sandbaken et al., 1990), leaving the N-terminal cassette intact. Indeed, our results show that both the basal and ribosome-stimulated ATPase activities were inhibited by trypsin cleavage, but the ribosome-stimulated activity was inhibited faster (Fig. 6). Kambampati and Chakraburty (1997) also observed the faster inactivation of the ribosome-stimulated activity; however, their results show that the basal activity was inhibited only 25%, when the ribosome-stimulated activity was inhibited 95%, and no intact protein remained. Contrary to our data, these results imply either that the N-terminal ATP binding site is more active in ATP hydrolysis than the C-terminal site, or that only the N-terminal binding site is active in ATP hydrolysis while the C-terminal ATP binding cassette has a role in EF-3 regulation.

Fig. 6. Effect of trypsin on ATPase activity of EF-3, shown on a (A) linear scale and (B) logarithmic scale. EF-3 was incubated with trypsin at a ratio of 50:1 at 22 °C for indicated periods, and aliquots were transferred to a tube containing soybean trypsin inhibitor (STI), to give ratio of STI to trypsin of 4:1. When all incubations were finished, EF-3 was assayed for basal ATPase activity (filled squares) and ribosome-stimulated activity (filled diamonds) by a coupled assay (see Materials and methods).

Additional research would be required to clarify the roles of the ATP-binding cassettes in ATP hydrolysis and EF-3 regulation.

The ratio of EF-3 to trypsin in these experiments was either 50:1 (Figs. 4–6) or 125:1 (not shown); therefore, the rate of ATPase inactivation is expected to be pseudo-first order. However, as shown in Figure 6, the rate of ATPase inactivation fits a second-order process. This result can be explained by three mechanisms: (1) there are two sites that are cleaved at different rates, either of which inactivates the ATPase; (2) various cleavages inactivate the ATPase to different extents; and (3) there are two populations of molecules, where one population is digested faster than the other. Our results (Figs. 4, 5) indicate that cleavages at various positions occur at different rates. This observation supports the first model. However, because both of the nucleotide binding sites of EF-3 are probably hydrolyzing ATP (Kovalchuk & Chakraborty, 1994; Yang et al., 1996), various cleavages may inactivate one or both of the ATPase sites to different extents. These experiments were performed with fresh samples, precluding the possibility that aggregates were present (Fig. 3). We do not have evidence, however, to support or reject the possibility of multiple monomeric forms being present.

Figure 5B shows that three regions of the molecule were relatively inaccessible to trypsin. These regions include the putative S5-homologous region (Belfield et al., 1995), the N-terminal ATP binding cassette, and the tRNA-synthase homologous region (Belfield et al., 1995). Based on this result and the supposed homology of the region between Lys102 and Ile368 to the prokaryotic ribosomal protein S5, we attempted to model the tertiary structure of this region after the known structure of *Bacillus stearothermophilus* S5 protein. However, our attempts to align these sequences using the Bestfit program (GCG package) gave an alignment that was different from the report by Belfield et al. (1995) (not shown). Homology between the EF-3–N-terminal domain and S5 can be inferred with greater confidence by using multiple alignments of several S5 and EF-3 sequences. Therefore, six S5 sequences were aligned with three EF-3 sequences using GCG's Pileup program. When complete EF-3 sequences were used, Pileup aligned the S5 sequences toward the carboxyl terminus of EF-3. Forcing the alignments to the amino terminus of EF-3 gave identities of only 12 to 18% between the S5 and EF-3 sequences. Furthermore, the alignment of Belfield et al. (1995) is based on a large number of small gaps in the S5 sequence, some of which are inserted in the middle of beta sheet elements in the S5 protein. Such insertions would disrupt those secondary structure elements, making the alignment arbitrary. Thus, we find no evidence to indicate that the EF-3 region from Lys102 to Ile368 is homologous to the bacterial S5 protein. Therefore, the proposed modeling is not possible.

This paper confirms a structural basis for the multidomain nature of EF-3. Based on the DSC data, it appears that when studied alone as a pure entity a component of the protein can undergo thermally induced unfolding at near-physiological temperatures. The proteolysis experiments suggest that this component is in the C-terminal one-third of the protein, because this domain was readily digested by trypsin. The loose structure of the C-terminal domain suggests that it can readily undergo conformational changes. Protection against heat inactivation by ribosomes and 30–100-fold stimulation of ATPase activity suggests that ribosomes induce a conformation change in the EF-3 protein. Furthermore, recent data from our laboratory show that ATP analogs greatly increase the binding of EF-3 to ribosomes (J. Harlan et al., unpubl. obs.), sug-

gesting that ATP may also induce a conformational change. Thus, it is reasonable to expect that these conformation changes would occur in the "loosely" structured C-terminus of EF-3. The N-terminal region of the protein was unusually resistant to proteolysis, suggesting it possesses a relatively tight structure. This protease-resistant domain may give rise to the thermal unfolding transition observed at 58 °C. The function of the N-terminal domain is not yet known. Unfortunately, it cannot be established by sequence homology analysis because this domain appears not to be homologous to any known protein.

Materials and methods

Protein purification

All steps were done at 4 °C. Yeast cell paste (75 g) containing an EF-3 overexpressing plasmid was added to 125 mL of lysis buffer [50 mM Tris(hydroxyethyl)aminomethane (Tris), pH 7.5, 10 mM MgCl₂, 10 mM β -mercaptoethanol, 30 mM KCl, 0.2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10% glycerol] and two tablets of Boehringer Mannheim Complete Protease Inhibitor. The mixture was homogenized for 3 min with 177 g of 0.5 mm glass beads in a Biospec homogenizer. The solution was centrifuged at 10,000 $\times$ g for 20 min and the supernatant was decanted. The pellet was re-washed with lysis buffer and centrifuged. The supernatants were combined and centrifuged at 24,000 $\times$ g for 30 min, and then at 61,500 $\times$ g for 120 min. The protein was precipitated using 70% (NH₄)₂SO₄ (saturation), and dialyzed against buffer A (10 mM Tris, pH 7.5, 10 mM MgCl₂, 10 mM β -mercaptoethanol, 0.2 mM EDTA, 10% glycerol). The pellet was used to isolate ribosomes as described elsewhere (Van Veldhoven & Mannaers, 1987).

The dialyzed supernatant was loaded onto a 300-mL QFF column at a flow rate of 2 mL/min. The column was washed with six column volumes of buffer A, and protein was eluted with a gradient of 0–750 mM KCl. EF-3-containing fractions were pooled, and the protein was precipitated with 70% (NH₄)₂SO₄ (saturation). The pellet was dialyzed against buffer B (10 mM Tris, pH 7.5, 15 mM KCl, 10 mM β -mercaptoethanol, 0.1 mM EDTA, 25% glycerol) and applied to a 60-mL S-Sepharose column at a flow rate of 2 mL/min. Protein was eluted with a 0–400 mM KCl gradient in nine column volumes of buffer B. EF-3-containing fractions appeared at about 200 mM KCl and were pooled. EF-3 yield was about 100 mg/L of culture grown in flasks. The protein molecular weight, checked by laser desorption mass spectrometry, was 116,600, which is in reasonable agreement with the calculated MW.

The molar absorptivity ($\epsilon_{\text{mole}}^{280}$) used in these experiments was 114,708 per mol (0.989 per 1 mg/mL) based on amino acid analysis, rather than the calculated value of 89,990.

Differential scanning calorimetry

Thermal stability of EF-3 was measured in a MicroCal MC-2 differential scanning calorimeter (DSC) after the samples were dialyzed into 50 mM NaHPO₄, pH 7.5, 10 mM MgCl₂, and 1 mM DTT.

Hydrodynamic measurements

Sedimentation velocity and equilibrium measurements were performed on a Beckman XL-A analytical ultracentrifuge and ana-

lyzed as described previously (Holzman & Snyder, 1994; Snyder et al., 1996). The experiments were carried out with EF-3 concentration of 1.0–1.4 mg/mL under the conditions indicated in Figure 3.

Cleavage with trypsin

EF-3 (1 mg/mL) was dialyzed into 30 mM Tris, pH 8.0, and 25% glycerol; trypsin was added to the dialyzed protein at a weight ratio of 50:1 EF-3 to trypsin and incubated at 22 °C. The reactions were stopped by transferring aliquots at the indicated times to tubes containing SDS sample buffer or 1% trifluoroacetic acid (TFA). SDS-PAGE was run with a BioRad mini slab gel apparatus according to the manufacturer's instructions.

RP-HPLC

Tryptic cleavage was performed as described above, except that a 30-min proteolysis reaction was stopped with TFA at a final concentration of 1%. The digest was loaded on an Altech C4 column (250 × 4.6, 5-μm particles, 300 Å pore size). Fragments were eluted with a linear H₂O/acetonitrile gradient of 0.5% acetonitrile/min, containing 0.1% TFA, at a flow rate of 0.5 mL/min. Fragments were collected and sequenced on an ABI 476 sequencer according to the manufacturer's instructions, or analyzed for molecular weight with a Brucker Reflex-II laser desorption mass spectrometer. LC-MS was carried out with a C18 ABI column (150 × 2.1 mm under the same conditions as above except that the flow rate was 100 μL/min) in line with a Finnigan MAT ESI-quadrupole TSQ-700 mass spectrometer.

ATPase assays

ATPase activity of EF-3 was assayed by a coupled assay to pyruvate kinase and lactic dehydrogenase as described in Sarthy et al. (1997) or by a modified malachite green procedure (Sarthy et al., 1997). Briefly, the coupled ATPase assay was performed at 30 °C in a final volume of 140 μL buffer consisting of 60 mM Tris pH 8.0, 5 mM Mg-acetate, 10 mM DTT, 10 mM KCl, 250 μM NADH, 1 mM phosphoenolpyruvate (PEP), 0.6 mM ATP, and 2 μL of pyruvate kinase/lactic dehydrogenase (PK/LDH; Sigma preparation P0294). Absorbance at 340 nm was followed for 5 min using a Molecular Devices (Menlo Park, California) plate reader. The malachite green assay was performed at room temperature in final volume of 30 μL consisting of 60 mM Tris-Cl, 20 mM NH₄Cl, 10 mM Mg-acetate, 10 mM DTT, 0.6 mM ATP, 0.05 units of ribosomes, and 0.27 μg EF-3. Reactions were incubated for 30 min and stopped by addition of 200 μL of color reagent (Van Veldhoven & Mannaers, 1987). Absorbance at 655 nm was read after 10 min.

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