Elongation factor 3 from the yeast Saccharomyces cerevisiae was purified over 230-fold from a high speed supernatant fraction. The homogeneity of the protein was shown by gel filtration and sedimentation equilibrium analysis of the native protein and by sodium dodecyl sulfate gel electrophoresis of the denatured protein. The molecular weight of the protein was estimated to be 125,000 by the above-mentioned methods. The protein consists of a single polypeptide chain. Amino acid analysis revealed no unusual features. Antibody raised against the purified factor showed a single cross-reacting band with the characteristic hexagonal pattern in an Ouchterlony double diffusion plate. The immune serum had no reactivity against the other two elongation factors (EF). The polymerization reaction was inhibited by the anti-EF3. Addition of excess EF3 could overcome this effect.

Factor 3 is absolutely required by the yeast ribosomes for polyphenylalanine synthesis. Ribosomes from other eukaryotes do not require this protein. The function of the third factor in polyphenylalanine synthesis cannot be defined at this time. The protein consists of a single polypeptide chain. Amino acid analysis revealed no unusual features. Antibody raised against the purified factor showed a single cross-reacting band with the characteristic hexagonal pattern in an Ouchterlony double diffusion plate. The immune serum had no reactivity against the other two elongation factors (EF). The polymerization reaction was inhibited by the anti-EF3. Addition of excess EF3 could overcome this effect.

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In vitro polypeptide chain elongation in eukaryotes require two well identified soluble protein factors. Elongation factor 1 participates in codon recognition by binding the correct aminoacyl-tRNA to the ribosome. Elongation factor 2 is required for the process of translocation. Eukaryotic elongation factor 1 is functionally analogous to the bacterial EF-Tu and the factor 2 is analogous to the bacterial EF-G.

Considerable information on the structure and function of eukaryotic soluble protein factors has come from studies with rat liver, rabbit reticulocyte, pig liver, Krebs ascites cells, brine shrimp, and wheat embryo (1). Although the yeast system provides a unique opportunity among eukaryotes to combine the methods of biochemistry and genetics for the analyses of the complicated processes of protein synthesis, this system has not been exploited to a great extent, perhaps because of the inability to obtain an active in vitro protein-synthesizing system from yeast. Recently, this limitation has been overcome by Gasior et al., who have prepared active cell-free system from yeast spheroplasts and have shown synthesis of intact proteins in response to exogenous messengers (2, 3). Prior to this, Richter and Klink, Richter and Lipmann, Spremulli and Ravel, and Torano et al., had isolated elongation factors 1 and 2 from yeast and had shown that they carry out functions corresponding to those of elongation factors from other eukaryotic sources (4-7). Skogerson isolated analogous factors from Saccharomyces cerevisiae (8). During the isolation procedure, he and his associates identified a third protein that they found to be uniquely required by the yeast ribosomes. Skogerson and Engelhardt showed that yeast ribosomes were completely inactive in poly(U)-dependent polyphenylalanine synthesis in the absence of the third factor. Ribosomes from liver, reticulocyte and from the brine shrimp Artemia salina were not influenced by this material. This yeast protein was referred to as elongation factor 3 by Skogerson (8). The current paper describes the purification of the yeast EF3, its amino acid composition, some of its functions, and its possible role in protein synthesis.

**Experimental Procedures**

**Results**

Factor 3 that is required by the yeast ribosomes for polyphenylalanine synthesis has been purified to homogeneity. Table I shows the yield in the peak fractions and the purification obtained in each step. The coincidence of the protein and the activity peak and the constant specific activity across the peak of the final Sephadex G-200 column (Fig. 1) are taken as criteria of purity. The protein was also found to be homogeneous by sodium dodecyl sulfate-gel electrophoresis (Fig. 2) and by sedimentation equilibrium analysis (Fig. 3). The upward curvature of the r vs. log concentration graph (Fig. 3) indicates a tendency of aggregation of this protein, although gel electrophoresis under non-denaturing conditions did not reveal this property (data not shown). The molecular weight of this protein calculated from the linear portion of the graph, and representing most of the material in the cell, was 110,000. Estimation of the molecular weight of the native enzyme by gel filtration (Fig. 4) and of the denatured protein by sodium dodecyl sulfate-gel electrophoresis (Fig. 2) using molecular weight markers gave a value of 125,000, indicating that this protein consists of a single polypeptide chain. Fig. 5

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shows the pattern of cross-reaction of EF3 in an Ouchterlony double-diffusion plate with the antiserum raised against the pure protein. Lack of cross-reaction of anti-EF3 with the elongation factors 1 and 2 (Fig. 5, wells 5 and 6), indicates that factor 3 is antigenically unrelated to either of the other two proteins.

Table II shows the amino acid composition of factor 3. There is no specific or unusual feature in the amino acid composition of the protein. In comparison to the elongation factor 1, the content of the hydrophobic amino acids and the basic amino acids are relatively low and the acidic amino acids are high in factor 3. The acidic isoelectric point of 5.9 is consistent with the amino acid composition.

Early experiments on a third factor requirement for polyphenylalanine synthesis by yeast ribosomes were done with partially pure EF3 that contained several contaminating proteins. Therefore, it was not possible to demonstrate whether the effect on polyphenylalanine synthesis was due to one or more components present in the preparation. With the homogeneous preparations of all three factors, it was possible to demonstrate unequivocally that, besides EF1 and EF2, the yeast ribosomes required one additional factor. With the homogeneous preparation of EF3, one could unequivocally demonstrate the dependence of the system on the addition of this protein. Fig. 6 shows dependence of the polymerization reaction on EF3 concentration and the effect of anti-EF3 antisera on the reaction. Poly(U)-dependent polyphenylalanine synthesis was completely inhibited by the immune serum. The inhibitory effect was overcome by the excess of EF3. Control serum had no effect on the system.

One possible explanation for the requirement of an additional factor by the yeast ribosomes could be that this protein results from the separation of one of the two elongation factors into two components. In that case, one would expect to see cross-reaction of anti-EF3 with EF1 or EF2. EF3 should also stimulate the partial reactions catalyzed by one of the two proteins. Lack of cross-reaction of EF3 antisera with EF1 and EF2 (Fig. 5) indicates that the proteins are not of the same origin.

A possible effect of the third factor on EF1 activity could be followed by measuring the stimulation of aminoacyl-tRNA binding to the ribosome. Similarly, an effect on EF2 activity could be measured as stimulation of EF2-dependent nucleotide binding to the ribosome in the presence of the antibiotic fusidic acid, which prevents recycling of EF2 (17). In a series of elegant experiments, Skogerson and Engelhardt had shown that EF3 does not affect the function either of EF1 or EF2 to a significant extent (10). Similar results were obtained by us with purified EF3. EF1-dependent binding of aminoacyl-tRNA to the ribosome is very rapid and is not greatly affected by temperature (1). A 2-fold stimulation of Phe-tRNA binding to the ribosome by EF3 at 0 °C and only at saturating concentrations of EF1 cannot explain the requirement of this factor for polyphenylalanine synthesis by the yeast ribosomes. In fact, liver ribosomes that do not require EF3 also showed similar stimulation by this protein (10).

We attempted to study the role of EF3 in peptide bond synthesis. Although EF1-dependent binding of aminoacyl-tRNA to the ribosomal A-site is temperature-independent, the peptide bond synthesis may be facilitated at a higher temperature. Therefore, the experiment was carried out at 30 °C instead of at 0 °C. However, the analysis of dipeptide synthesis was complicated by the presence in the ribosome preparation, sufficient amount of EF1 and EF3 to carry out the complete elongation cycle. This difficulty was overcome by including anti-EF2 in the assay along with the ribosome and the factors. As can be seen in Fig. 7, addition of EF3 or EF1 + EF3 to the ribosome resulted in increased binding of [14C]Phe-tRNA to the nitrocellulose membrane, but the extent of binding was reduced by a large factor when anti-EF3 was added in the system. We analyzed the products from both sets of experiments by alkali hydrolysis of the membrane-bound radioactivity (17) and by paper chromatography (18) of the total and the ethyl acetate-extractable products (19). The analyses showed the formation of large oligomers in the absence of anti-EF2. When anti-EF2 was present in the reaction mixture along with EF1 and EF3, the bulk of the nitrocellulose-bound product, upon alkali extraction and subsequent paper chromatography, appeared as free amino acid and not as dipeptide (data not shown). From this experiment, we concluded that the stimulatory effect of EF3 on EF1-dependent Phe-tRNA binding to the ribosome is not due to enhanced rate of dipeptide synthesis but reflects increased binding of the aminoacyl-tRNA to the ribosome.

Elongation factors 2 and 3 both show ribosome-dependent GTP hydrolysis independent of each other. The rate of GTP hydrolysis by EF3 is 10 times faster than by EF2. However, EF2 has higher affinity for GTP (K_m = 10 μM). Moreover, GTPase activity of EF2 was 80% inhibited at a substrate concentration of 60 μM (data not shown). The K_m for EF3 for GTP is much higher (125 μM) and the reaction was not inhibited by high GTP concentrations. Whether GTP hydrolysis by EF3 plays any role in peptide elongation needs further experimentation.

Factor 3 also has strong ATPase activity. In fact, the rate of ATP hydrolysis was 3 times faster than the rate of GTP hydrolysis. The K_m for ATP is 55 μM. Neither EF1 nor EF2 showed ATPase activity. Both GTPase and ATPase activities of EF3 were inhibited by the immune serum at comparable concentrations and were expressed after the addition of excess of EF3 (Figs. 8 and 9).

The process of elongation involves recycling of the factors. Elongation factor 1 forms a ternary complex with GTP and aminoacyl-tRNA that binds to the ribosome. In bacterial systems, GTP is hydrolyzed and Tu-GTP is released after delivering the aminoacyl-tRNA to the P-site of the ribosome. A low molecular weight protein EF Ts functions in recycling of EF Tu by the following exchange reaction:

\[ \text{EF} \text{TuGDP} + \text{GTP} \rightleftharpoons \text{EF} \text{TuGTP} + \text{GDP} \]

A protein analogous to bacterial EF Ts has been reported to occur in eukaryotic systems (1). This protein, EFβ, required for the polymerization reaction, stimulates the binding of Phe-tRNA and the nucleotide exchange reactions with limiting amount of EF1α. The protein EFβ has often been isolated in association with EF1α. In yeast we have as yet no indication of the existence of EF1β or a high molecular weight form of EF1α. Elongation factor 3, which is uniquely required by the yeast ribosomes, does not stimulate the nucleotide exchange reaction (data not shown). On the basis of this experiment and the results discussed earlier, we conclude that EF3 does not correspond in function to EF1β.

In the bacterial system, one of the ribosomal proteins, S1, has been shown to dissociate from and reassociate with the ribosome very easily. Poly(U)-dependent polyphenylalanine synthesis is stimulated by this protein and the translation of natural message is almost completely dependent on the addition of S1. Khanh et al. (21) have shown that the protein S1 binds strongly to synthetic and natural messages. The requirement of EF3 by the yeast ribosomes and not by the liver ribosomes indicates that this protein could be an easily dissociable ribosomal protein like the bacterial S1. Therefore, we tested the poly(U)-binding property of EF3 and checked the cross-reactivity of EF3 with the antiserum to the bacterial S1.
Purification and Properties of the Yeast Elongation Factor 3

(Kindly performed by A. Subramanian of the Max Planck Institute, West Germany). The binding of poly(U) to EF3 was very weak and there was little cross-reaction of this protein with S1 antisera (data not shown). This indicates that yeast EF3 is very probably not a ribosomal protein analogous to the bacterial S1 in function.

DISCUSSION

The unusual requirement of a third protein factor by the yeast ribosomes for poly(U)-directed polyphenylalanine synthesis prompted us to characterize this protein and to study its function in the elongation cycle.

Although the elongation factors 1 and 2 are interchangeable with homologous factors from other eukaryotic sources, factor 3 is not required by ribosomes other than those isolated from yeast. It is possible that factor 3 is species-specific and, therefore, does not function in heterologous systems. Indeed, antisera raised against EF3 did not affect the polymerization reaction by the liver ribosomes. Another possibility is that EF3 is a loosely bound ribosomal protein and is removed easily during the isolation procedure from yeast ribosomes whereas liver ribosomes and the ribosomes from other sources are saturated with an analogous protein and are unaffected by this factor. An unlikely possibility is that yeasts evolved differently and have a unique requirement for an additional soluble protein factor to carry out the elongation process.

Skogerson and Engelhardt had studied some of the partial reactions to determine which of the three elongation cycle reactions was affected by EF3. Our results with homogeneous EF3 preparation is in good agreement with the results obtained by them with impure preparations. As previously discussed, EF3 was not required for the EF1- and GTP-dependent binding of Phe-tRNA. The difference in the reaction observed by us between 0°C and 30°C could be accounted for by the stimulation of the polymerization reaction at 30°C due to contaminating amounts of the other two factors present in the ribosome. The 2-fold stimulation of Phe-tRNA binding by EF3 at 0°C with saturating concentrations of EF1 was due to increased binding of Phe-tRNA to the ribosome. No effect of EF3 was seen in the EF2-dependent binding of GDP to the ribosome. In the presence or absence of fusidic acid (9). Ribosome-dependent GTPase activity of EF2 did not require the presence of EF3 although EF3 itself has a much stronger ATPase activity and do not require the addition of EF3 for the polymerization reaction. A possible correlation of the ATP effect, the ATPase activity of EF3, and its requirement by the yeast ribosomes is under current investigation.

The translation of synthetic messages generally does not require initiation factors although polymerization must be initiated by some mechanism. Wahba et al. had shown a stimulatory effect of initiation factor 3 on poly(U)-directed polyphenylalanine and poly(A)-directed polylysine synthesis with Escherichia coli ribosome (23). The possibility that the newly discovered elongation factor is actually an initiation factor cannot be ruled out at this point.

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K. Chakraburtty and B. Dasmahapatra, unpublished results.
Purification and Properties of the Yeast Elongation Factor 3

Experimental Procedures

Materials: Biochemicals and reagents were obtained from standard suppliers. Triticale and tRNA preparations, radiolabeled amino acids and nucleosides were obtained from New England Nuclear. Female New Zealand white rabbits were obtained from Lyle M. Wallis, Department of Animal Science, University of Wisconsin. Quanlified ribosomes were prepared by Dr. B. Rehner's laboratory of Syacuse University. Yeast, S. cerevisiae, strains were obtained from Dr. D. Davis, Department of Biophysics, Wisconsin and also from Bion Laboratory, Geneva, Switzerland.

Growth of Cells and Preparation of Cell Extract: Saccharomyces cerevisiae strain 7782 was grown inYP medium at 30°C with agitation to a cell density of 10^6 cells/ml. The cells were disrupted under milder conditions than previously described (13). This disrupted cell extract was centrifuged at 20,000 x g for 20 min at 4°C. The supernatant was collected by continuous flow centrifugation. The precipitated protein was collected by centrifugation at 20,000 x g for 20 min at 4°C and dialyzed overnight against the same buffer containing 0.5 M KCl.

Isolation of Ribosomes: Ribosomes were isolated by centrifugation at 4°C in sucrose buffered with 10 mM Tris. The ribosomes were disrupted at 4°C with 1 volume of 100 mM spermidine and 0.1 M MgCl2. The ribosomes were disrupted under milder conditions than previously described (13). This disrupted cell extract was centrifuged at 20,000 x g for 20 min at 4°C. The supernatant was collected by continuous flow centrifugation. The precipitated protein was collected by centrifugation at 20,000 x g for 20 min at 4°C and dialyzed overnight against the same buffer containing 0.5 M KCl.

Isolation of phenylalanyl-tRNA synthetase. Charged trpPhe after the polymerization reaction and GTPase activity eluted toward the gradient.

Deacylase activity determined by the deacylation of several factors. Previous studies using other methods have shown that these factors were pure.

Results

Table I

| Fraction | Number of Cells | Total Activity | Specific Activity | Purification |
|----------|----------------|---------------|-----------------|--------------|
| Size 200| 1 x 10^9        | 100           | 1               | -            |
| DIA/Aephaedes | 6 x 10^8        | 545           | 9.0             | 1            |
| CM-Sepharose | 3 x 10^8        | 19           | 6.3             | 1            |
| Hydroxyapatite | 2 x 10^7        | 105          | 5.2             | 1            |
| Sephadex | 1.5 x 10^7       | 255          | 16.7            | 1            |

Values of amino acids were determined by spectrophotometry to zero time hydrolysate. Cysteine was determined as cysteinylcysteine after incubation with 0.01 M Tris-HCl pH 7.5 containing 1 mM PMSF. Values of amino acids were determined by the method of Dubois et al. (15). Amino acids were determined by the method of Dubois et al. (15). Amino acids were determined by the method of Dubois et al. (15).
Purification and Properties of the Yeast Elongation Factor 3

Figure 1. Sedimentation equilibrium analysis of the elongation factor 3. The analysis was performed in a Beckman model E ultracentrifuge at 3°C and at 14,000 rpm for 48 hours.

Figure 2. SDS-polyacrylamide gel electrophoresis of purified yeast EF3. Lane A contained 10μg of purified EF3, lane B contained 2μg of purified EF3. Molecular weight markers: Hemoglobin (67,000), Alkaline phosphatase (115,000), Galactosidase (115,000), Phosphorylase b (97,000), Bovine serum albumin (67,000), Ovalbumin (45,000). 8 μg of purified EF3 was run in well A and 10 μg of purified EF3 was run in well B.

Figure 3. Molecular weight determination of the native EF3. The molecular weight of the purified factor 3 preparation was determined by gel filtration on a Sephacryl S-300 column. The molecular weights of the calibration proteins determined by specific assays for individual proteins.

Figure 4. Counter-current immunodiffusion analysis of EF3. The immune serum was applied to the centre well. Purified EF3 (10μg) was applied in a radial manner. Well 1 contained 10μg of EF3 and well 2 contained 5μg of EF3.
Purification and Properties of the Yeast Elongation Factor 3

Figure 4. The effect of anti-ES3 on Polyubiquitin Synthesis. The contents of reaction mixtures and conditions of incubation were as described in assay procedures except that the amount of EF1 was as indicated in the Fig. Equivalent amount of pre-immune serum was used in experiments with pre-immune serum (D—D) and immune serum (A—A).

Figure 5. Effect of ES3 on phe-tRNA binding activity of EF1. The equivalent conditions were as described in the experimental procedures except that the phe-tRNA was used at indicated times and washed through the immobilin disc membranes. EF1 + anti-ES3 (A—A); EF1 + ES3 + anti-ES3 (A—A); EF1 alone (A—A); ES3 + anti-ES3 (A—A).

Figure 6. The effect of anti-ES3 on the ribosome-dependent hydrolysis of ATP. The contents of reaction mixtures and the conditions of incubation were as described in assay procedures except that the amount of protein (5 ug/sample) was used in experiments with pre-immune serum (D—D) and immune serum (A—A).

Figure 7. The effect of anti-ES3 on the ribosome-dependent hydrolysis of ATP. The experimental conditions are as described in the previous figure.