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Elongation Factor 1 from Krebs II Mouse Ascites Cells

Purification, Structure and Enzymatic Properties

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A procedure for the purification of elongation factor 1 (EF-1) from Krebs II mouse ascites cells is described. Ascites EF-1 occurs in multiple forms of different molecular weight like the corresponding enzymes from calf brain and rat liver. Biogel A-5m chromatography of our purest EF-1 resulted in a pattern indicating the presence of different molecular weight components. However, only a single polypeptide band was observed when this same material was analysed by electrophoresis on acrylamide gels containing sodium dodecylsulphate. With this method the molecular weight of the EF-1 polypeptide chain was determined to be 47000. It is suggested that the occurrence of multiple forms of EF-1 is due to the association of 47000-mol. wt subunits to form aggregates of different sizes.

The factor has essentially the same enzymatic properties which were described for EF-1 from reticulocytes, calf brain and rat liver. The attachment of aminoacyl-tRNA to ribosomes catalyzed by EF-1 follows a strict 1:1 stoichiometry. EF-2 when introduced into the ribosomal binding assay allows the recycling of EF-1 and subsequent synthesis of polypeptide chains. The possible implications of this finding with respect to the conditions which allow stoichiometric or catalytic function of EF-1 are discussed.

Elongation factor 1 functions in polypeptide synthesis by catalyzing the attachment of aminoacyl-tRNA to the ribosomal acceptor site [1,2]. This reaction has been shown to require GTP and some evidence has recently been produced suggesting the formation of a ternary complex between aminoacyl-tRNA, GTP and EF-1 as a necessary intermediate in the binding of aminoacyl-tRNA to the ribosomal A-site [3,4]. It has also been shown that the interaction of the aminoacyl-tRNA anticodon with the complementary codon is a prerequisite for the cleavage of those GTP molecules which became attached to the ribosomal A-site as part of this ternary complex [3]. Apart from this specific GTP hydrolysis into GDP and phosphate, an uncoupled GTPase activity is displayed by EF-1 in the presence of ribosomes and aminoacyl-tRNA [5,6], the functional significance of which is not known.

In spite of the advances which have recently been made in understanding the function of EF-1, many questions pertaining to the structure as well as the function of this protein remain to be resolved. McKeehan and Hardesty [7] have purified the EF-1 activity from rabbit reticulocytes to homogeneity as judged by ultracentrifugation and dodecylsulphate-acrylamide-gel electrophoresis. They found their elongation factor to consist of three identical subunits each having a molecular weight of 62000. The molecular weight of the active protein was measured by ultracentrifugation as being 186000 [7]. In contrast to these results, other authors have found multiple forms of EF-1 in various organs [6,8,9]. In calf brain, the EF-1 activity could be detected in protein fractions with a molecular weight ranging from 50000 to 1.5×10^6 [3]. Data concerning the subunit structure or the function of these fractions have, so far, not become available.

Nothing is known on the recycling of EF-1 subsequent to its attachment to the ribosomal acceptor site. The search for a EF-Ts-like activity which plays a critical role in recycling EF-Tu in bacteria has only produced negative evidence.

In view of these open questions a systematic investigation of EF-1 in a cell system which has lent itself easily to the study of mammalian protein biosynthesis seemed warranted. We have therefore purified EF-1 from Krebs II ascites cells. Here we are reporting on the procedure of purification, the molecular weight, the subunit structure and some enzymatic properties of this protein.

Abbreviations. EF-1, elongation factor 1; EF-2, elongation factor 2; EF-Ts, elongation factor Ts; EF-Tu, elongation factor Tu; Guo-5′-P_2-CH_2-P, 5′guanylyl-methylene-diphosphonate.

Definition. A_{260} unit, the quantity of material contained in 1ml of a solution which has an absorbance of 1 at 260 nm when measured in a 1-cm path-length cell.

EXPERIMENTAL PROCEDURE

Chemical

[^{3}H]Phenylalanine (specific activity, 5 Ci/mmol); [^{14}C]phenylalanine (specific activity, 400 mCi/mmol) and [γ-^{32}P]guanosine 5′-triphosphate ammonium salt (specific activity > 10 Ci/mmol) were purchased from the Radiochemical Centre (Amersham, England). Poly(uridylic acid), creatine phosphate, creatine kinase, phospho*enol*pyruvate and phospho*enol*pyruvate kinase were obtained from Boehringer Mannheim GmbH (Mannheim, Germany). Guanosine triphosphate (GTP), sodium dodecylsulphate, as well as all reagents needed for the acrylamide-gel electrophoresis and the standard proteins for the molecular weight determinations came from Serva Laboratories (Heidelberg, Germany). Biogel A-5m was a product of the Biorad Laboratories (New Jersey, U.S.A.). Calcium phosphate gel came from Sigma Chemicals (St. Louis, Missouri, U.S.A.). Preparation of calcium hydroxyapatite followed the procedure given by Bernardi [10]. All other chemicals came from Merck KG (Darmstadt, Germany) and were of the highest degree of purity available.

Cells and Media

Krebs II ascites tumor cells were maintained in National Medical Research Institute mice by weekly intraperitoneal transfer.

The following media were used in the preparation of elongation factor 1 (EF-1) and ribosomes.

Medium A: 0.25 M sucrose, 0.125 M KCl, 0.030 M Tris-HCl pH 7.5, 6 mM 2-mercaptoethanol, 5 mM $(CH_3COO)_2Mg$.

Medium B: 0.3 M sucrose, 0.100 M KCl, 0.050 M Tris-HCl pH 7.5, 1 mM dithiothreitol, 0.1 mM EDTA.

Medium C: 0.3 M sucrose, 75 mM KH_2PO_4, 75 mM K_2HPO_4 pH 7.0, 1 mM dithiothreitol, 0.1 mM EDTA.

Medium D: 0.05 mM Tris-maleate pH 7.5, 1 mM dithiothreitol, 0.1 mM EDTA.

Ribosome buffer [11]: 0.2 M sucrose, 0.1 M NH_4Cl or KCl, 0.05 M Tris-HCl pH 7.6, 5 mM $(CH_3COO)_2Mg$ and 1 mM dithiothreitol.

ASSAYS

Ribosomal Binding Assay

The enzymatic binding of aminoacyl-tRNA to ribosomes was carried out in 0.1-ml assays containing: 5 mM $(CH_3COO)_2Mg$, 100 mM KCl, 0.05 mM Tris-HCl pH 7.5, 1 mM glutathione, 0.2 mM GTP, 2 A_{260} units washed ribosomes, 10 pmol [^{3}H]Phe-tRNA^{Phe}, 20 μg poly(uridylic acid), 0.1—1 μg purified EF-1 or varying amounts of EF-1-containing fractions. Incubations were carried out at 37 °C and lasted 15 min. Other technical details were as described previously [12].

Whenever the possibility existed that EF-1 preparations were still contaminated with EF-2, the latter component was inactivated by means of *N*-ethylmaleimide treatment as reported by McKeehan and Hardesty [7].

Protein Biosynthesis

Assays were carried out in 0.1-ml volumes containing: 80 mM KCl, 50 mM Tris-HCl pH 7.6, 6 mM 2-mercaptoethanol, 1 mM ATP, 0.6 mM CTP, 0.1 mM GTP, 5.5 mM Mg^{2+}, 10 mM creatine phosphate and 20 μg creatine kinase. Each tube also contained 0.5 or 1 μg pure EF-1, 10 μg partially purified EF-2, 2 A_{260} units ribosomes, 50 pmol ^{3}H or ^{14}C-labeled phenylalanine-tRNA^{Phe} and 20 μg poly(uridylic acid). Terminations of the reactions, alkaline and acid hydrolysis, filtration and assessment of radioactivity were carried out as reported previously [12].

[^{32}P]GTPase Assay

The GTPase activity of EF-1 was measured in the absence and presence of ribosomes, phenylalanyl-tRNA and poly(U) according to Conway and Lipman [13].

Protein concentrations were determined as described by Lowry *et al.* [14].

Molecular-Weight Determinations

The molecular weight of various EF-1 preparations was estimated by gel filtration on standardized A-5m Biogel columns as described by Andrews [15, 16].

The determination of the molecular weight of single peptide chains (EF-1 subunits) by dodecylsulphate-acrylamide-gel electrophoresis followed the instructions given by Weber and Osborn [17]. Instead of bromphenol blue we used myoglobin (molecular weight, 17200) as an internal standard for assessing the mobility of EF-1 and standard proteins since bromphenol blue never gave a sharp band and therefore did not allow as precise a calculation of the mobility of a given protein as did myoglobin.

Acrylamide-Gel Electrophoresis

Electrophoresis of native proteins on acrylamide gels was performed according to Davis [18]. All gels were stained with coomassie brilliant blue and destained electrophoretically.

PREPARATIVE PROCEDURES

Ribosomes

For the isolation of ribosomes, Krebs II ascites cells were harvested from mice which had been

inoculated 7 days prior to sacrifice. The cells were washed 3 times in ice-cold 0.146 M NaCl, 0.035 M Tris-HCl pH 7.6 buffer and pelleted by centrifugation at $2000 \times g$. Two additional washes in hypotonic buffer (10 mM KCl, 10 mM Tris-HCl pH 7.6 and 5 mM $[CH_3COO]_2Mg$) were then performed to remove traces of erythrocytes and to swell the ascites cells. A volume of the same hypotonic buffer equal to that of the swollen cells was added to the cell pellet after the last centrifugation. 20-ml portions of the cell suspension were then transferred to a Dounce homogenizer and broken by three strokes with a tight-fitting pestle. Isotonicity was immediately restored in the homogenate by the addition of an appropriate volume of 1.25 M KCl, 0.3 M Tris-HCl pH 7.6, 60 mM 2-mercaptoethanol and 50 mM $(CH_3COO)_2Mg$ buffer. The total homogenate was then centrifuged for 15 min at $30000 \times g$ and the resulting supernatant recentrifuged for another 15 min at the same speed. The extract was made 1 mM in ATP, 0.6 mM in CTP, 0.1 mM in GTP and 10 mM in creatine phosphate. Creatine kinase was added to a final concentration of 150 to 200 μg/ml of the extract and a mixture of 20 amino acids was introduced to a final concentration of 50 μM for each amino acid. After a 60 min incubation at 37 °C a fibrous precipitate which had formed during the incubation was removed with a glass rod or by centrifugation at $15000 \times g$ for 10 min. The extract was then layered over a step gradient consisting of 7 ml 1.8 M sucrose and 7 ml 1 M sucrose in 0.1 M NH_4Cl, 0.02 M Tris-HCl pH 7.6, 5 mM $MgCl_2$ and 1 mM dithiothreitol. The "run-off" ribosomes were obtained by centrifugation at 50000 rev./min in the 60 Ti rotor of the Spinco L-2-65 B centrifuge for at least 8 h. Each ribosome pellet was dissolved in 2.5 ml ribosome buffer. The ribosomal suspension was then made 0.5 M with respect to NH_4Cl by the dropwise addition of an appropriate volume of the same buffer containing 2.0 M NH_4Cl. Washed ribosomes were recovered by centrifugation over a 14-ml cushion of 20% sucrose in 0.5 M NH_4Cl ribosome buffer. This washing procedure was repeated twice. The final preparations were dissolved in ribosome buffer at a concentration of 150 to 200 A_{260} units/ml and stored under liquid nitrogen. For a number of experiments in which it seemed critical to eliminate any EF-2 or non-specific nucleoside triphosphate phosphohydrolase activities remaining on the ribosomes after three washes, the ribosomes were treated with 0.05 M *N*-ethylmaleimide prior to use [7].

Elongation Factor 2

EF-2 was isolated as described by Felicetti and Lipman [19]. This preparation contained no EF-1 as judged by its complete inability to support the synthesis of polyphenylalanine in the absence of EF-1.

PURIFICATION OF ELONGATION FACTOR 1

Preparation of the Extract

Krebs II ascites cells from 100—200 mice were harvested, washed 3 times in Hanks balanced salt solution and suspended in a volume of medium A corresponding to 2.5 times the weight of the packed cells. 100-ml portions of the cell suspension were then disrupted by 5 bursts from a Bransone sonifier B-12, each burst lasting 10 s with the output-control in position 7. The intervals between the single sonications lasted 5—10 s. The resulting homogenate was centrifuged at $30000 \times g$ for 30 min in a refrigerated centrifuge. The upper four-fifths of the homogenate were gently aspirated with a pipette. A thin lipid-layer floating on top of the homogenate was left behind together with the sediment.

pH-5.2 Fractionation and Ammonium-Sulphate Precipitation

1 M acetic acid was added dropwise to the homogenate under constant stirring until the pH had reached 5.2. The precipitate was removed by a 25-min centrifugation at $20000 \times g$. The clear supernatant was immediately brought back to pH 7.2 by the addition of an appropriate amount of 1 M KOH.

A differential $(NH_4)_2 \cdot SO_4$ precipitation was performed next. The material precipitating between 40 and 70% saturation was collected and dissolved in 50 to 100 ml medium B.

Calcium-Phosphate-Gel Fractionation

After extensive dialysis of the crude factor preparation against several liters of medium B and removal of precipitated material by low-speed centrifugation, 12 g calcium phosphate gel for each gram protein were suspended in a volume of medium B corresponding to 3 times the weight of the gel. The protein solution was slowly added to the slurry under constant stirring and allowed to adsorb to the gel for another 30 min. The gel was then recovered by a 5 min centrifugation at $10000 \times g$ and washed three times in medium B. The fraction containing most of the EF-1 activity was obtained from the gel by elution with 0.3 M potassium phosphate buffer after removal of EF-2 and other proteins with 0.15 M buffer as described by Moldave [20].

Biogel Chromatography

The material eluted from the calcium phosphate gel by 0.3 M phosphate was precipitated with $(NH_4)_2 \cdot SO_4$ (70% saturation), redissolved in medium B to yield a concentration of approx. 25 mg protein/ml, and dialyzed for 12 h against several changes of the same buffer.

Subsequently, the material was applied to a Biogel A-5m column equilibrated with medium B. For

the fractionation of 350 to 400 mg total protein a column (5 × 95 cm) was used. In general, 40 to 50 ml of gel bed were needed for every 10 mg protein. The active fractions eluting from the Biogel A-5m column were pooled, concentrated by vacuum dialysis or by precipitation with $(NH_4)_2 \cdot SO_4$ and applied to a hydroxyapatite column.

Hydroxyapatite Chromatography

This step was carried out essentially as described by McKeehan and Hardesty [7]. However a continuous 150 to 400 mM potassium phosphate gradient (in medium C) was used for the elution of the factor instead of the step procedure described by the above authors. The amounts of protein applied to the column never exceeded 2 mg/ml bed volume. The active fractions from the hydroxyapatite column were pooled and concentrated by vacuum dialysis against medium D which was made 10% in sucrose for this purpose.

Sucrose-Gradient Electrophoresis

The final purification of EF-1 was achieved by preparative electrophoresis on a 20—60% sucrose gradient. This step was carried out in a U-shaped glass apparatus constructed in this institute. It has an internal diameter of 1.4 cm and is equipped with a cooling jacket. Each arm of the U is topped with an electrode buffer vessel and one arm of the U has a capillary inserted for the fractionation of the gradient. The lower part of the apparatus was filled with 70% sucrose in medium D. A 30-ml 20—60% sucrose gradient was then slowly layered onto the 70% sucrose cushion while the other arm was simultaneously filled with electrode buffer (medium D without sucrose) such that the system was kept in complete balance. The protein samples (2—6 mg in 1—2 ml 10% sucrose in medium D) were applied to the top of the gradient, another layer of 5% sucrose in medium D was layered over the sample to protect it from minor turbulences and both electrode buffer vessels were simultaneously filled with medium D. Proteins were allowed to migrate into the gradient (cathode to anode) for 48—72 h at 1200 volts, and 3.5 mA. At the end of the run, the gradient was stabilized by closing one arm of the U-shaped apparatus. It was then pumped through an ultraviolet-monitoring device attached to a recorder and fractionated.

RESULTS

Extent of Purification

Table 1 summarizes the results of a typical purification experiment and demonstrates that a more than 600-fold enrichment of EF-1 was achieved after the last step of the procedure. The greatest loss in total activity (75%) seems to occur during the chromatography on Biogel A-5m. The magnitude of this loss, however, can be explained by the fact that EF-1 elutes from the Biogel column in several peaks, representing components of different molecular weights and that only the major peak corresponding to a molecular weight in the range of 240000 to 280000 was used for further purification. A substantial amount of total activity represented by fractions of lower molecular weight was therefore deliberately excluded from Table 1. Sodium dodecylsulphate-acrylamide gels of EF-1-containing fractions at various stages of purification are shown in Fig. 1 (gels 1—5). The final preparation (Fig. 1, gel no. 5) contains a dense single polypeptide band and only minute amounts of contaminating proteins. The material which appears as a single polypeptide band in dodecylsulphate gels is also found as a single component on 7% urea gels (Fig. 1, gel no. 6) and can therefore be regarded as being homogeneous not only in size but also in charge.

Table 1. *Purification of EF-1 from Krebs II ascites cells*
146 g cells were processed as described under Experimental Procedures. One unit of EF-1 was defined as the activity which catalyzed the transfer of 1 picomole phenylalanyl-tRNA^{Phe} to ribosomes under the conditions of the ribosomal binding assay described above. All fractions except the last two were pretreated with *N*-ethylmaleimide as described by McKeehan and Hardesty [7] to destroy any EF-2 and prevent synthesis of polyphenylalanine

Fraction	Volume	Protein		Total activity	Specific activity	Purification
			Total			
	ml	mg/ml	mg	10^{-3} units	units/mg protein	-fold
Extract	600	16.5	9900	167.0	17.0	1.0
pH 5.2	588	7.3	4292	161.0	37.5	2.2
40—70% $(NH_4)_2 \cdot SO_4$	38.5	49.2	1894	151.0	80.0	4.7
Calcium phosphate gel	7.5	16.4	123	84.0	690.0	40.6
Biogel A-5m (peak A)	2.2	4.4	9.8	21.0	2197	129.0
Hydroxyapatite	1.0	2.15	2.15	9.3	4189	234.0
Sucrose-gradient electrophoresis	0.5	1.5	0.79	9.1	11509	618.0

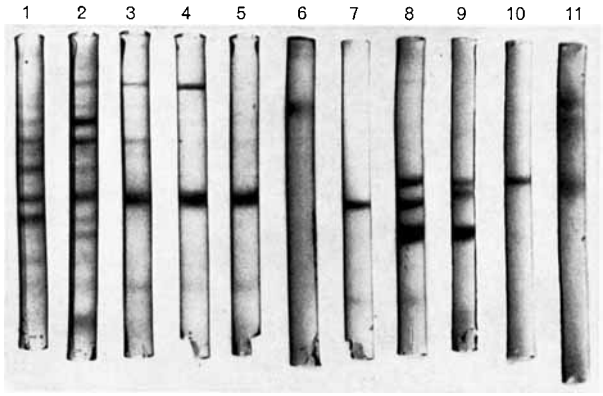

Fig. 1. *Electrophoretic separation of EF-1 preparations from various stages of purification and of standard proteins on 5×54 mm acrylamide gels.* Electrophoresis was carried out at 80 volts using a current of 10 mA per tube. Gels 1—5, and 7—9 were 7% (w/v) with respect to acrylamide and contained 0.1% (w/v) sodium dodecylsulphate. Gels 1—5 and 7—9 were run in parallel. Gels 6, 10, and 11 are from separate individual runs. Gel (1) 20 μg 40—70% $(NH_4)_2 \cdot SO_4$ precipitate after dialysis; gel (2) 16 μg of material after calcium phosphate gel fractionation; gel (3) 9 μg heavy component from Biogel A-5m column; gel (4) 9 μg EF-1 after purification on hydroxyapatite; gel (5) 8 μg EF-1 after final purification by sucrose-gradient electrophoresis; gel (6) 8 μg of the same purified EF-1 (as in gel no. 5) in 7% acrylamide gels containing 8 M urea; gels (7—9) comparative mobility of EF-1 polypeptide with standard proteins. 3 μg EF-1 were applied to gel 7, the same amount of EF-1 plus 5 μg aldolase (molecular weight, 40000) and 5 μg catalase (molecular weight, 60000) were applied to gel 8. Identical amounts of aldolase, catalase and human gamma-globulin (heavy chain) were run on gel 9. Gels (10, 11) Electrophoretic analysis of purified EF-1 on sodium dodecylsulphate acrylamide gel and a 4% acrylamide gel without sodium dodecylsulphate. Gel (10) 5% acrylamide gel containing 0.1% sodium dodecylsulphate, 3.2 μg EF-1. Gel (11) 70 μg of the same EF-1 preparation was applied to a 4% acrylamide gel and subjected to electrophoresis for 2 h in 50 mM Tris-maleate pH 7.5, 1 mM dithiothreitol and 0.1 mM EDTA

Multiple Forms

EF-1 preparations from rat liver and calf brain have been shown to occur in multiple forms corresponding to different molecular weights. As already mentioned, this finding is also made with EF-1 from Krebs II ascites cells. When EF-1 is separated from the bulk of EF-2 by stepwise elution from calcium phosphate gel and is subsequently chromatographed on the Biogel A-5m column, the aminoacyl-tRNA binding activity is eluted from the column over a large number of fractions representing a wide range of molecular weights. A typical elution profile obtained from a column which had previously been standardized with several proteins of known molecular weight [15, 16] is shown in Fig. 2. The major components within this wide distribution range correspond to estimated molecular weights of >260000, 230000 and 135000. Fig. 3 summarizes the molecular weight obtained with several factor preparations in different experiments. Since the heaviest component (250000 to 280000 mol.wt) always represented a major part of the total binding activity which was eluted from the columns and moreover showed the highest activity/protein ratio, this material was commonly used for further purification. Surprisingly, the purified EF-1 derived from this "heavy" material contained comparatively little activity corresponding to this high molecular weight when rechromatographed on a Biogel A-5m column. The main component was now determined to have a molecular weight of 180000 to 190000 and a substantial proportion of the total activity appeared to be even lighter (Fig. 4).

Molecular Weight of the EF-1 Polypeptide Chain

When EF-1 purified 600-fold was subjected to acrylamide electrophoresis under denaturing condi-

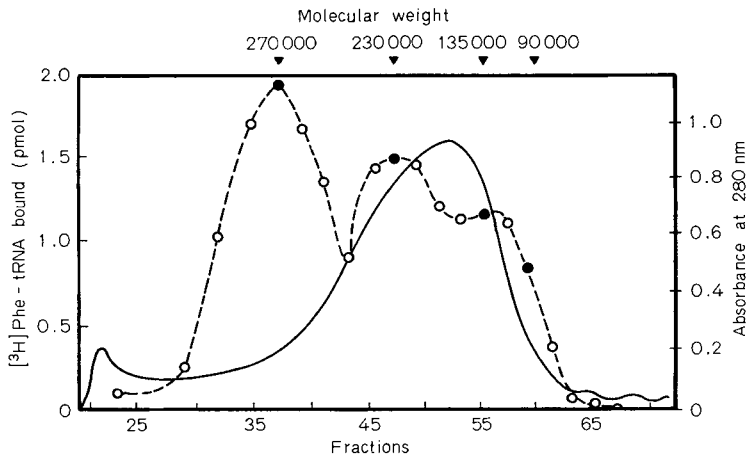

Fig. 2. *Elution profile of EF-1 from an A-5m Biogel column.* 27 mg of a factor preparation which had passed the calcium phosphate gel step were applied to a Biogel A-5m column (2.7×75 cm) equilibrated with medium B. The material was then eluted at a constant flow rate of 0.63 ml/min. Fractions of 6.25 ml were collected and 10-μl aliquots of every third fraction were treated with *N*-ethylmaleimide and assayed for EF-1 activity in a typical ribosomal binding assay. (——) Absorbance at 280 nm; (O----O) elution profile of EF-1 activity; (●) fractions corresponding to the molecular weights indicated

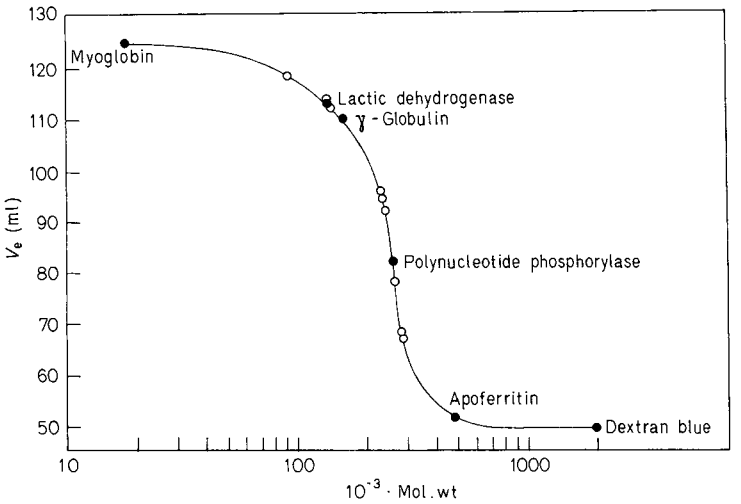

Fig. 3. *Determination of the molecular weight of several EF-1 preparations with standard proteins.* All preparations as well as the standard proteins were chromatographed separately on a Biogel A-5m column (2.7×75 cm) at a constant flow rate. The elution volumes (V_e) of standard proteins or of EF-1 activity peaks were plotted against a semilogarithmic molecular weight scale. (●) Donate the positions of standard proteins; (○) EF-1 preparations. The EF-1 peak having a molecular weight of 90000 and one peak corresponding to a molecular weight of 190000 were from purified preparations, the other peaks represent preparations after the calcium phosphate step

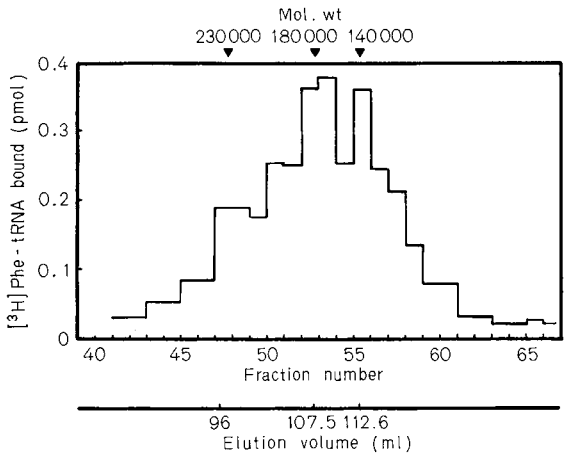

Fig. 4. *Elution profile of a highly purified EF-1 preparation, derived from a heavy (>260000 mol.wt) fraction.* 100 µg EF-1 purified by sucrose-gradient electrophoresis were applied to a Biogel A-5m column (1.6×88 cm) equilibrated with medium B and eluted at a constant flow rate of 0.25 ml/min. 40-µl aliquots from each fraction (2.03 ml) were introduced into a typical ribosomal binding assay for the assessment of EF-1 activity. The column had previously been standardized with the proteins mentioned in Fig. 3

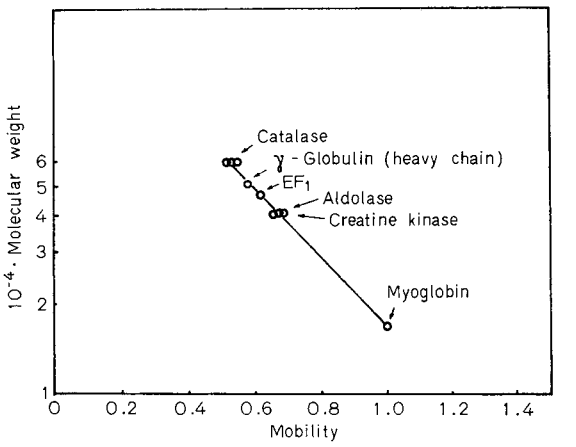

Fig. 5. *Molecular-weight determination of the EF-1 polypeptide in acrylamide gels containing 0.1% (w/v) sodium dodecylsulphate.* All proteins were run on duplicate gels. The runs for catalase, aldolase and EF-1 were repeated three times on 5×54 mm gels and twice on 5×100 mm gels. The extrapolated molecular weight value for the EF-1 band is 47000

tions on 7.5% acrylamide gels in 0.1% sodium dodecylsulphate, all the protein moved as a single polypeptide band (Fig. 1, gel no. 7). The location of this band was assayed in relation to a number of standard proteins of known molecular weight [17]. Gel number 8 in Fig. 1 shows the EF-1 polypeptide located between a 40000 (lower band) and a 60000 (upper band) molecular weight standard. For comparison, the relative positions of standard peptides with molecular weights of 60000, 50000 and 40000 are shown on gel number 9 (from top to bottom) which was run in parallel. From the location of the factor band in relation to a number of standard peptides, a molecular weight of 47000 can be assigned to the EF-1 polypeptide chain (Fig. 5).

Structure of EF-1

In view of the homogeneity of the single polypeptide in sodium dodecylsulphate gels as well as in urea gels, the most purified native EF-1 with an estimated molecular weight of 180000 to 190000 could be regarded as a tetrameric structure consisting of four identical polypeptide chains each having a molecular weight of 47000. Although the purified factor, the elution profile of which is shown in Fig. 4, contains only a single polypeptide chain as judged by electrophoresis in sodium dodecylsulphate or in urea-acrylamide gels, it elutes from a Biogel column as a broad peak representing a wide range of molecular weights. This phenomenon can best be explained by the ability of the 47000-mol.wt subunit to form polymers of different multiplicity. As a matter of fact, the active fractions appearing in the elution profiles of Biogel A-5m columns with remarkable regularity had molecular weights of approximately 90000, 130000, 190000, 230000 and 280000. These fractions could all be regarded as multiple forms of a subunit consisting of a single polypeptide chain weighing 47000 daltons. While an active fraction with an estimated molecular weight of 80000 to 90000 was a frequent, if not regular, finding we never detected activity in the range of 50000 molecular weight. This may indicate that the 47000-mol.wt subunit is by itself inactive.

Biogel fractions corresponding to molecular weights of 90000 or 140000 proved less stable on further purification than the fractions corresponding to higher molecular weights. When the purified EF-1 preparations derived from heavy (>260000) or light (140000) crude EF-1 fractions were compared with respect to their molecular weights by rechromatography on a standardized Biogel A-5m column, it turned out, that the majority of the heavy EF-1 material now eluted from the Biogel A-5m column at a position corresponding to a molecular weight of 190000 while the light material yielded a preparation with an estimated molecular weight of 90000. A small but reproducible difference between these two EF-1 preparations became evident when they were compared with respect to their electrophoretic mobility on sucrose gradients. The purified EF-1 (188000 mol.wt) migrated slightly ahead of the EF-1 (90000 mol.wt). As can be seen in Fig. 6 there is almost no cross-contamination between the two preparations. It is noteworthy, however, that the heavy preparation after gradient electrophoresis has about twice the specific activity of the light material. Attempts to demonstrate the differences in the electrophoretic mobility of heavy and light EF-1 complexes using acrylamide gels were only partially successful: very soft gels (4% acrylamide) and longer time periods had to be used in order to allow the heavy EF-1 preparation to enter the gel. This caused rather diffuse bands. It is, however, clear from Fig. 1, gel

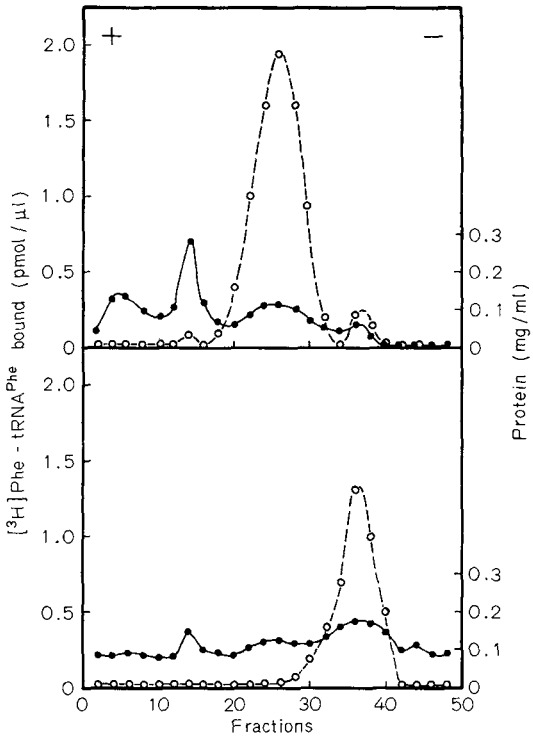

Fig. 6. *Sucrose-gradient electrophoresis of purified EF-1 preparations derived from heavy (>260000-mol.wt) and light (140000-mol.wt) components of EF-1.* Both preparations were taken through all purification steps including chromatography on hydroxyapatite. Fractions of 0.85 ml were collected from both gradients. The average molecular weight of the heavy material was 190000 after purification on hydroxyapatite, that of the light purified EF-1 was 90000 at the same stage. 3.6 mg of the heavy material and 4.0 mg of the light EF-1 were applied to identical sucrose gradients as described in the experimental section. The samples were run in parallel in two identical glass apparatus attached to the same power supply and to the same cooling machine for 62 h. Migration was from right to left. After fractionation, protein concentrations and [^{3}H]Phe-tRNA^{Phe}-binding activities were measured in every second fraction. Protein concentrations are indicated by the solid circles (●). The open circles (○) represent the binding activity present in 1-µl aliquots

no. 11, that a purified EF-1 preparation derived from the heavy fractions from Biogel A-5m column gave three diffuse bands in acrylamide gel and that the same material upon electrophoresis in 7.0% acrylamide gel containing 0.1% sodium dodecylsulphate yielded a single band with the properties already described (Fig. 1, gel. no. 10).

Properties of Purified EF-1

Fig. 7 illustrates that very low concentrations of purified EF-1 promote the binding of [^{3}H]phenylalanyl-tRNA^{Phe} to ascites ribosomes washed in NH_4Cl and treated with *N*-ethylmaleimide prior to

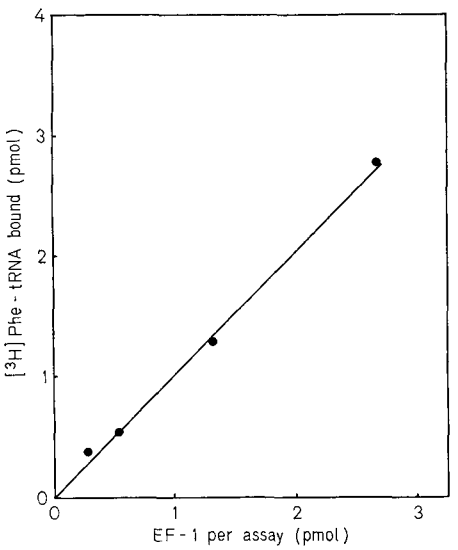

Fig. 7. *Relationship between the attachment of [^{3}H]Phe-tRNA^{Phe} to ribosomes and the amount of EF-1 present in the assay mixture.* The binding assays were carried out as described under Experimental Procedures. The average molecular weight of the EF-1 preparation was 188000. The filled circles (●) indicate the amounts of Phe-tRNA^{Phe} bound to ribosomes in the presence of 0.05, 0.1, 0.25 and 0.5 μg EF-1

use. Assuming an average molecular weight for this EF-1 preparation of 188000, 0.5 μg pure material would correspond to 2.65 pmol factor. The introduction of this amount of EF-1 into the incubation mixture allowed the attachment of 2.79 pmol phenylalanyl-tRNA. Thus, the molar ratio between EF-1 in the system and aminoacyl-tRNA becoming attached to ribosomes is close to 1. This result would indicate that even in the presence of excess ribosomes, Phe-tRNA^{Phe} and GTP, the factor functions stoichiometrically and is not capable of recycling. The question as to which conditions would allow EF-1 to function catalytically during protein synthesis has as yet not been answered but is presently under investigation in this laboratory.

The same amount of purified EF-1 which promoted the binding of aminoacyl-tRNA to the ribosomal A-site at a ratio of 1:1 could very effectively support the synthesis of polyphenylalanine when EF-2 was supplied (Table 2). It is shown that the ribosomes used in this study were virtually free of elongation factors (after *N*-ethylmaleimide treatment) since no phenylalanine synthesis is observed until both factors are present. Under the conditions described in the legend of Table 2 and in the experimental section the presence of an energy-regenerating system was not necessary for optimal incorporation of phenylalanine.

Lin *et al.* [5] have shown that EF-1 from reticulocytes becomes more heat-stable in the presence of

Table 2. *Synthesis of polyphenylalanine supported by EF-1*
The conditions of the assay were as described in the experimental section. 0.5 μg of the same EF-1 preparation used in the binding assay depicted in Fig. 7 were introduced into each tube

Conditions of incubation	Phenylalanine incorporated
	pmol
Complete	14.1
— EF-1	0.3
— EF-2	0.6
— EF-1, EF-2	0.4
— Energy-regenerating system, ATP, CTP	14.6
— GTP	0.3

Table 3. *[γ-^{32}P]GTP cleavage by EF-1 from ascites cells*
The assays were carried out in 100-μl volumes containing 0.3 M sucrose, 0.2 M KCl, 1 mM dithiothreitol, 0.1 mM EDTA, and 1 mmol [γ-^{32}P]GTP. Where indicated, ribosomes, phenylalanyl-tRNA^{Phe} and poly(U) were present at concentrations of 40 pmol, 33 pmol and 20 μg respectively per assay. 7 μg (approximately 37 pmol) of EF-1 were introduced into each tube

Additions	[γ-^{32}P]GTP, cleaved
	pmol
Ribosomes	4
EF-1	15
Ribosomes, Phe-tRNA^{Phe}	3
Ribosomes, EF-1	27
EF-1, Phe-tRNA^{Phe}	27
Ribosomes, Phe-tRNA^{Phe}, EF-1	607
poly(U), EF-1	17
poly(U), ribosomes, EF-1, Phe-tRNA^{Phe}	491

aminoacyl-tRNA and GTP but that the stability of factor is not positively influenced by the presence of GTP alone. Analogous results were obtained for EF-1 from ascites tumor cells as shown in Fig. 8. The increased heat stability of EF-1 in the presence of GTP and aminoacyl-tRNA could be taken as indirect evidence for the formation of a ternary complex between the three reactants. Our attempts to demonstrate the existence of such a complex more directly by filtration of a corresponding reaction mixture through Sephadex G-150 as described by Collins *et al.* [6] have so far been unsuccessful.

Similar to EF-1 from reticulocytes [5, 7] and rat liver [6], EF-1 from ascites cells displayed very little GTPase activity when incubated with [γ-^{32}P]GTP. The addition of ribosomes or aminoacyl-tRNA alone caused only a very slight increase of this activity. If, however, these constituents were introduced into the reaction mixture simultanously, very rapid hydro-

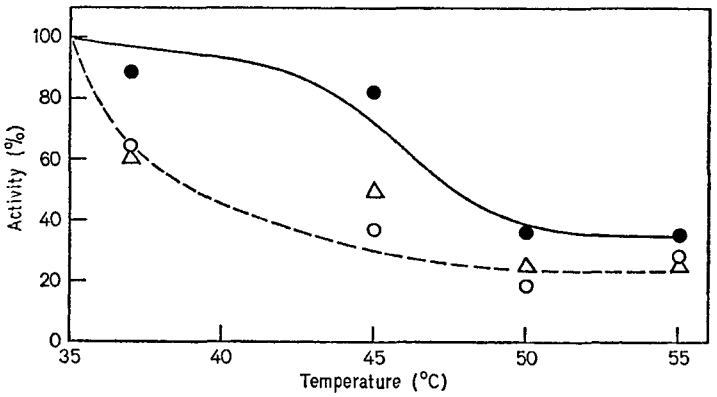

Fig. 8. *The effects of GTP and aminoacyl-tRNA on the heat stability of purified EF-1 from ascites cells.* EF-1 was heated for 5 min at the indicated temperatures with or without GTP and aminoacyl-tRNA and then used to carry out ribosomal binding at 37 °C in the typical assay (see Experimental Procedures). Activity is expressed as a percentage of that observed without heat inactivation. (○) EF-1 alone; (△) 2.5 pmol EF-1 + 0.4 mM GTP; (●) 2.5 pmol EF-1 + 0.4 mM GTP + 100 pmol of unfractionated mouse tRNA (charged with all amino acids except phenylalanine)

lysis of GTP ensued. Under these conditions, approx. 100 mol GTP were hydrolysed for each mol EF-1 during an incubation period of 20 min (Table 3). The addition of poly(uridylic acid) to the reaction mixture had no marked influence on the rate of GTP hydrolysis. Weissbach *et al.* [3] have recently presented evidence in favour of a 1:1 relationship between aminoacyl-tRNA attached to ribosomes by EF-1 from calf brain and GTP cleaved in this reaction. According to their results the hydrolysis of GTP occurs when the aminoacyl-tRNA bound to the ribosomes as part of a ternary complex together with EF-1 and GTP interacts with the complementary codon. It is, therefore, doubtful whether the "uncoupled" reaction described here and previously observed by others [5, 6] has any physiological meaning. It may merely represent an artificial reaction occurring under conditions *in vitro*. However, it offers an additional means of describing the purified enzyme and should therefore not go without mention.

DISCUSSION

The purification procedure for EF-1 from Krebs II ascites cells as used in this study followed the general outline of purification as already described by others [6, 7, 20, 21]. Some of the data which are compiled in Table 1, however, require comment.

The absorption to and stepwise elution from a batch of calcium phosphate gel, first introduced by Moldave [20] for the separation of rat liver EF-1 from EF-2, was ommitted from most recent procedures in favour of a final or prefinal purification step on a hydroxyapatite column [6, 7]. However, the data shown in Table 1 indicate that the fractionation with calcium phosphate gel is the most effective single step of the whole purification sequence and that it does not preclude the use of a hydroxyapatite column at a later stage. The purification against the previous step (gel filtration) achieved by hydroxyapatite chromatography is 1.6-fold in the procedure published by Collins *et al.* [6] without previous fractionation on calcium phosphate gel. In our study the corresponding step still achieved a 1.8-fold purification against the previous gel filtration inspite of the use of calcium phosphate gel at an earlier stage of the purification procedure. Since hydroxyapatite and calcium phosphate gel do have different crystal structures [10, 22], this finding seems not at all surprising. McKeehan and Hardesty used a sucrose-gradient centrifugation as final step in the purification of EF-1 from rabbit reticulocytes [7], while Collins *et al.* [6] used electrofocusing in their purification procedure for EF-1 from rat liver. Preparative sucrose-gradient electrophoresis as employed in this study appears to combine the advantages of both methods without being burdened with their shortcomings. The sucrose-gradient centrifugation only allows a 1.5 times increase in the specific activity of EF-1 [7]. Electrofocusing, on the other hand, is a powerful tool of purification but is also characterized by a loss of total activity as high as 90% [6]. These rather discouraging results which were essentially confirmed in our laboratory led us to introduce a preparative electrophoresis on a sucrose gradient as the last purification step. This method causes almost no losses but allows an almost 3-fold rise in the specific activity of the final preparation above the value obtained for the EF-1 from the hydroxyapatite column.

One of the most puzzling features of EF-1 from rat liver and calf brain has been the occurrance of

these enzymes in multiple forms relating to widely different molecular weights. Weissbach *et al.* [3] have separated EF-1 fractions from calf brain into three groups on the basis of molecular weight; a high-molecular-weight fraction of about 1.5×10^6, a medium fraction containing molecular weights between 300000 and 850000 and a low-molecular-weight fraction containing species of between 50000 and 125000. Collins *et al.* [6] have recently described EF-1 fractions ranging from molecular weight of 170000 to 400000 as judged by their elution from Sepharose columns. These authors also noted a tendency of the heavy fractions to become smaller upon further purification. EF-1 species varying considerably in size are also found in Krebs II ascites cells. We would like to propose that the existence of multiple forms of EF-1 in Krebs II ascites tumor cells is due to the formation of complexes between varying numbers of identical subunits each having a molecular weight of 47000. Although our data do not lend themselves to a rigorous proof of this hypothesis, they provide strong evidence to support it. In the first instance, there can be no doubt that pure EF-1 preparations can be derived from material eluting from the Biogel column in a molecular weight range of >260000 as well as from fractions representing lower molecular weights and that these pure or nearly pure preparations of EF-1 appear to contain but one major polypeptide band with a molecular weight of 47000. Secondly, the existence of EF-1 multimers of various sizes also becomes evident if one compares the elution pattern of purified EF-1 in Fig. 4 or the formation of several diffuse bands by the same factor in a 4% acrylamide gel with the homogeneous band which this material displays in acrylamide gels containing 0.1% sodium dodecylsulphate. Thirdly, the EF-1 binding activity in the elution profile from Biogel A-5m columns is not evenly distributed around one central fraction with a maximal activity but occurs in several distinct peaks which are found with remarkable regularity in different preparations of EF-1 and the molecular weight of which can be regarded as multiples of 47000. Further work will be necessary to provide definite proof for this hypothesis and to explain the functional meaning of the postulated association—dissociation phenomena involving the 47000-mol.wt subunit.

An interesting question arises from the observation that purified EF-1 bound Phe-tRNA at a molar ratio of 1:1 in the binding experiment depicted in Fig. 7, but proved capable of catalytic function as soon as EF-2 was provided. Stoichiometric function of EF-1 from rat liver and from rabbit reticulocytes was also observed by other authors [5, 6]. There appear to be at least two possible explanations for this phenomenon: it is conceivable that EF-1 is bound to the ribosomal A-site together with GTP and aminoacyl-tRNA during the binding reaction and that it is not released from this site unless EF-2 is present. The second possibility would be that after the interaction of the bound tRNA with the complementary codon and the ensuing GTP hydrolysis [3] EF-1 is released on the ribosome as EF-1 · GDP which would have to undergo an exchange reaction with GTP or some other kind of "regeneration" in order to become available for carrying a new aminoacyl-tRNA to the ribosomal acceptor site.

The first explanation seems attractive because the addition of EF-2 to a typical aminoacyl-tRNA binding assay like the one described in Fig. 7 will lead to polyphenylalanine synthesis which in view of the low concentrations of EF-1 present in this assay must involve recycling of this elongation factor. However, since the EF-2 preparations used in this study, though free of EF-1, are not pure, we cannot at present exclude the possibility that a protein other than EF-2 is responsible for the recycling of EF-1. With respect to the second possibility it should be noticed that the addition of creatine kinase and phosphocreatine or of phospho*enol*pyruvate and the corresponding kinase do not appear to alter the function of EF-1 [6] (and Grasmuk and Drews, unpublished results). This negative result does not of course completely exclude the existence of a protein analogous in function to EF-Ts in bacteria but it appears to argue against this possibility. Attempts are presently being made in our laboratory to discriminate between the two alternatives mentioned above by following the fate of labeled EF-1 during the binding reaction and the subsequent translocation step. The results of these studies will be presented in due time.

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