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Multiple Forms and Some Properties of Aminoacyltransferase I (Elongation Factor 1) from Rat Liver†

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ABSTRACT: Elongation factor 1 (aminoacyltransferase I), the factor involved in binding aminoacyl-tRNA to ribosomes, has been extensively purified from rat liver. On a Sepharose 6B column the factor elutes in two peaks, one of which is larger and one smaller in molecular weight than elongation factor 2 (aminoacyltransferase II). The smaller is relatively unstable, but has been separated from elongation factor 2 and its enzymic activities partially characterized. The material eluting toward the front of the column contains active species of approximately 400,000 and 170,000 molecular weight, as determined on sucrose gradients. After further purification of the larger components through isoelectric focusing, there are two forms differing in isoelectric point, both of which are active in amino acid incorporation, binding of aminoacyl-tRNA to ribosomes, and ribosome-dependent GTP hydrolysis. Each of these can be shown to contain additional multiple forms by phosphocellulose chromatography and disc gel electrophoresis. Each is heterogeneous in size with a range of of 60,000–170,000 molecular weight indicating a decrease in size during purification. The most highly purified enzyme is catalytic in promoting amino acid incorporation and ribosome-dependent GTP hydrolysis, but probably not in binding aminoacyl-tRNA to ribosomes. Amino acid incorporating activity can be stimulated under certain conditions by pyruvate kinase and phosphoenolpyruvate. The binding activity of elongation factor 1 can be inhibited by elongation factor 2.

Polypeptide chain elongation in mammalian cells consists of three steps (Skogerson and Moldave, 1968a): the binding to the ribosome of the aminoacyl-tRNA corresponding to the codon being read, which binding is promoted by elongation factor 1 (aminoacyltransferase I)[1]; the formation of a peptide bond between the peptidyl chain in the donor site on the ribosome and the newly bound aminoacyl-tRNA, promoted by peptidyl transferase, a component of the large ribosomal subunit; and translocation of the newly lengthened peptidyl-

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[1] Abbreviations used are: GDPCP, the β–γ-methylene analog of GTP; EF-1, elongation factor 1 (transferase I, aminoacyltransferase I, T_1); EF-2 elongation factor 2 (transferase II, aminoacyltransferase II, T_2); p*I*, isoelectric point. In the uniform nomenclature recently proposed for the factors involved in protein synthesis, the factor formerly called aminoacyltransferase I was designated EF-1 for elongation factor 1 and aminoacyltransferase II EF-2 for elongation factor 2.

tRNA from the acceptor to the donor site, catalyzed by elongation factor 2 (aminoacyltransferase II). We have previously reported studies on purified EF-2 from rat liver (Raeburn *et al.*, 1971; Collins *et al.*, 1971b). In the present report we describe further purification and some properties of EF-1 from the same source. EF-1 has been purified to homogeneity from rabbit reticulocytes (McKeehan and Hardesty, 1969) and partially purified from rat liver (Gasior and Moldave, 1965; Schneir and Moldave, 1968; Hradec *et al.*, 1971; Gasior *et al.*, 1971), calf liver (Klink *et al.*, 1967), and calf brain (Moon and Weissbach, 1972). We have been unable to obtain a homogeneous preparation of EF-1 from rat liver, partly because the enzyme is heterogeneous in size, as reported by Schneir and Moldave (1968), and shows in addition multiple forms on disc gel electrophoresis, phosphocellulose chromatography, and isoelectric focusing. The most purified enzyme from rat liver is similar to less pure preparations from rat liver and to the reticulocyte enzyme in the activities which it promotes: complementation with EF-2 for polypeptide chain elongation on ribosomes, the binding of aminoacyl-tRNA to ribosomes in the presence of GTP and mRNA, and a GTPase dependent on ribosomes and aminoacyl-tRNA. The preparation is catalytic in promoting amino acid incorporation into protein and ribosome-dependent GTP hydrolysis, but probably acts stoichiometrically in the binding of aminoacyl-tRNA to ribosomes in the absence of EF-2.

In bacterial systems two separable factors, EF-Ts and EF-Tu, are required for the binding of aminoacyl-tRNA to ribosomes during polypeptide chain elongation (Lucas-Lenard and Lipmann, 1971). One of these, EF-Ts, can be replaced by phosphoenolpyruvate and pyrvuate kinase (Weissbach *et al.*, 1970). In the present report we show that the activity of EF-1 in polypeptide chain elongation can be stimulated by pyruvate kinase and phosphoenolpyruvate, and that some forms of EF-1 appear to be more active than others in the binding of aminoacyl-tRNA to ribosomes. However, the total activity of any two forms of EF-1 together is never more than additive, and there is no indication of a factor similar to EF-Ts in mammalian systems.

Our studies demonstrate that the binding of aminoacyl-tRNA to ribosomes, (both enzymatic binding facilitated by EF-1 and nonenzymatic binding), is inhibited by highly purified EF-2. These observations can be interpreted to indicate overlapping sites for the two factors EF-1 and EF-2 on the ribosome. A number of recent observations in bacterial systems have been interpreted in a similar manner (Cabrer *et al.*, 1972; Miller, 1972; Richman and Bodley, 1972; Richter, 1972).

Experimental Section

Materials. Many of the materials used were the same as described previously (Raeburn *et al.*, 1971; Moon *et al.*, 1970). Hydroxylapatite (Hypatite C) was a product of Clarkson Chemical Co.; Sepharose 6B was from Pharmacia; Whatman P-11 cellulose phosphate and microgranular DE-52 preswollen DEAE-cellulose were purchased from H. Reeve Angel. Ampholine carrier ampholytes were from LKB Instruments, Inc.

Poly(U) was purchased from Miles Laboratories. *Escherichia coli* L-[^{14}C]leucyl-tRNA (0.355 μCi of [^{14}C]leucine/mg of aminoacyl-tRNA), *E. coli* L-[^{14}C]phenylalanine tRNA (0.18 μCi of [^{14}C]phenylalanine/mg of aminoacyl-tRNA), and [γ-^{32}P]GTP (6–8 Ci/mmole) were from New England Nuclear.

Ribosomes both with (Raeburn *et al.*, 1971) and without (Moon *et al.*, 1970; Martin and Wool, 1969) endogenous mRNA and peptidyl-tRNA were used.

Methods

Assays. ELONGATION FACTOR 2. EF-2 was determined by the incorporation of [^{3}H]ADP ribose from [^{3}H]NAD into EF-2 catalyzed by diphtheria toxin (Raeburn *et al.*, 1971) or by complementation with EF-1.

ELONGATION FACTOR 1. EF-1 activity was determined in a coupled assay with EF-2 and ribosomes, measuring the amount of radioactive amino acids from aminoacyl-tRNA incorporated into trichloroacetic acid precipitable polypeptides, and by stimulation of the binding of [^{14}C]phenylalanyl-tRNA to ribosomes in the presence of poly(U). The incorporation assay using [^{14}C]leucyl-tRNA and ribosomes containing endogenous mRNA and peptidyl-tRNA has been described (Raeburn *et al.*, 1971). A second incorporation assay using poly(U), [^{14}C]Phe-tRNA, and ribosomes free of endogenous mRNA and peptidyl-tRNA contained, in a volume of 0.25 ml: 10 mM $MgCl_2$, 60 mM NH_4Cl, 10 mM dithiothreitol, 50 mM Tris-HCl (pH 7.2, at 25°), 0.6 mM GTP, 0.05 mg of poly(U), 0.01 μCi (26 pmoles) of [^{14}C]Phe-tRNA, and 0.1–0.2 mg of ribosomes EF-1 and EF-2. The EF-2 used was the highly purified preparation from either the phosphocellulose or electrofocusing steps (Raeburn *et al.*, 1971). After incubation for 10 min at 37°, the reaction mixture was treated as described previously for the [^{14}C]leucyl-tRNA assay (Raeburn *et al.*, 1971).

The binding of [^{14}C]Phe-tRNA to ribosomes was done in a manner similar to that described previously for Met-tRNA binding (Moon *et al.*, 1970). The 0.25-ml assay mixture contained 8 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.2), 60 mM NH_4Cl, 10 mM dithiothreitol, 0.06 mM GTP, EF-1, 0.1–0.2 mg of ribosomes (free of endogenous mRNA and peptidyl-tRNA), 0.02 mg of poly(U), and 0.01 μCi of [^{14}C]Phe-tRNA. GDPCP replaced GTP in some experiments.

GTPase Assay. The GTPase activity of purified EF-1 was assayed as described previously (Raeburn *et al.*, 1971) except that in this case EF-1 and aminoacyl-tRNA were used. The reaction mixture contained, in the volume of 0.125 ml: 50 mM Tris-Cl (pH 7.2, at 25°), 5 mM $MgCl_2$, 80 mM NH_4Cl, 57 mM mercaptoethanol, 15 μg of aminoacyl-tRNA, approximately 0.1 mg of ribosomes, 4.4 μM [γ-^{32}P]GTP (approximately 0.02–0.05 μCi), and enzyme as indicated. Incubation was for 30 min at 37°. To a portion of the material not absorbed onto Norit was added 10 ml of Aquafluor (New England Nuclear) and the sample was counted in a Nuclear-Chicago Mark I scintillation counter with an 80% efficiency for ^{32}P. Initially 0.1–0.125 mg of unlabeled aminoacyl-tRNA was added per reaction to enable detection of the GTPase. Since commercial preparations were variably active, it was found more reliable to use tRNA acylated with [^{14}C]phenylalanine plus 19 [^{12}C]amino acids. The amount of ^{14}C used gave negligible spill into the ^{32}P channel.

Gel Electrophoresis. Polyacrylamide gels were prepared as described previously (Raeburn *et al.*, 1971) except that only three-fourths the usual amount of the acrylamide solution was used in order to allow EF-1 to enter the gel. The separating gel contained 5.2% acrylamide and 0.14% bisacrylamide. To assay EF-1, the separating gels were sliced into approximately 40 sections as described previously (Collins *et al.*, 1971b) and slices were soaked, two together in sequence from the top, in 0.15 ml of 1 mg/ml of serum albumin in buffer A (0.05 M Tris-Cl (pH 7.2, 25°)–10^{-3} M dithiothreitol–

10^{-4} M EDTA). After soaking overnight in a refrigerator, the fluid in the tubes was assayed as described above. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was done by the method of Weber and Osborn (1969).

Sucrose Gradients. The size distribution of enzyme in various steps of enzyme preparation was determined on linear 10–30% (w/v) sucrose gradients. A 0.52-ml sample of enzyme in buffer A, 0.2 mg of pyruvate kinase (Sigma) and in some cases 1.5–3.0 mg of bovine serum albumin, was layered on a linear gradient of 10–30% sucrose (Mann Enzyme Grade) in buffer A, then centrifuged for 48–72 hours at 22,000–24,000 rpm in an SW 25.1 swinging-bucket rotor. Fractions (1 ml) were collected with an Isco density gradient fractionator. Fractions were analyzed for serum albumin, pyruvate kinase, and EF-1. Molecular weights were estimated after the method of Martin and Ames (1961), using $s_{20,w}$ = 10.1 S and mol wt 235,000 for pyruvate kinase, and $s_{20,w}$ = 4.32 S and mol wt 68,000 for bovine serum albumin.

Purification of Elongation Factor 1. Livers (average weight of 6 g) were excised from 40 Sprague-Dawley female rats (100–150 g weight each) and chilled in Littlefield's medium (0.25 M sucrose, 25 mM KCl, 5 mM $MgCl_2$, and 0.05 M Tris-Cl, pH 7.2). The following steps were done at 0–5°. The livers were homogenized two at a time in 15 ml of Littlefield's medium with a motor-driven tissue grinder (Thomas, No. 4288-B, size C). The homogenates were pooled and the preparation was centrifuged for 5 min at 4000*g* in a Servall GSA rotor, followed immediately by centrifugation for 40 min at 13,000*g*. The supernatant fraction was called the postmitochondrial supernatant fluid. The supernatant fraction was brought to pH 5.1 by the gradual addition of 1 M acetic acid, then centrifuged at 13,000*g* for 20 min. The supernatant fluid was brought to pH 7.0 by the addition of 1 M KOH. Powdered ammonium sulfate was gradually added to this solution to 40% saturation (22.6 g/100 ml). The preparation was stirred for 30 min, then centrifuged at 13,000*g* for 20 min. To the supernatant fraction was added more ammonium sulfate to produce 70% saturation (18.2 g/100 ml). The preparation was stirred and then centrifuged as above. The precipitate was dissolved in buffer A and dialyzed overnight against buffer A (1 l. followed by a 1-l. change). After dialysis the enzyme solution was centrifuged for 5 min at 20,000*g*. If a floating precipitate was present in the supernatant fraction, it was removed by aspiration. The supernatant fluid was then centrifuged at 105,000*g* for 2 hr in a Beckman-Spinco 30 rotor. The supernatant fraction was used for subsequent steps. Without the ultracentrifugation step, the flow rate on Sepharose 6B was appreciably slowed.

Column Chromatography. SEPHAROSE 6B. The dialyzed enzyme solution (65 ml) was chromatographed on a 4.5 × 53 cm column of Sepharose 6B, equilibrated with buffer A. The column was developed with buffer A at a flow rate of 80 ml/hr. Fractions (10 ml) were collected. The fractions from the first peak of EF-1 activity were pooled and dialyzed overnight against 0.01 M potassium phosphate (pH 7.0), containing 1 mM dithiothreitol (1 l. with one or two 1-l. changes).

HYDROXYLAPATITE. The pooled, dialyzed solution was loaded onto a 1.8 × 7.4 cm column of hydroxylapatite, and eluted with a 500- or 600-ml linear gradient of 0.01–0.5 M potassium phosphate (pH 7.0), containing 1 mM dithiothreitol. Fractions (8 ml) were collected at a flow rate of 25 ml/hr. Fractions at the peak of the enzyme activity were pooled, concentrated, and dialyzed overnight against 0.01 M Tris-Cl (pH 7.2), 10^{-3} M dithiothreitol, and 10^{-4} M EDTA in preparation for isoelectric focusing.

CM (Carboxymethyl)-Sephadex Chromatography. Alternatively in an attempt to reduce the amount of precipitate on the electrofocusing column, the pooled enzyme from hydroxylapatite chromatography was dialyzed against two or three 500-ml changes of 0.04 M potassium phosphate (pH 6.2), containing 1 mM dithiothreitol, 2 mM $MgCl_2$, 20% glycerol, and chromatographed on a 3 × 5.4 cm CM-Sephadex C-50 column equilibrated with the same buffer. The enzyme was absorbed to the column and was eluted with a 300-ml linear gradient of 0.04–0.8 M potassium phosphate (pH 6.2), containing dithiothreitol, $MgCl_2$, and glycerol. At least two peaks of activity were seen on such columns. These two peaks were not separately characterized. The most active fractions from both peaks were combined and dialyzed against 0.01 M Tris-Cl (pH 7.2), 1 mM dithiothreitol, and 0.1 mM EDTA, in preparation for electrofocusing.

Isoelectric Focusing. The sample was prepared for isoelectric focusing by the addition of 2% (w/v) of pH 3–8 ampholytes, a mixture of equal parts of pH 3–6 and 5–8 ampholytes. If a precipitate formed after ampholyte addition, it was removed by centrifugation at 17,000*g* for 5–15 min. This precipitate, which was soluble in buffer A, sometimes contained a large amount of EF-1 activity. The formation of this precipitate could be reduced by making the enzyme solution approximately 50% sucrose before addition of ampholytes. The supernatant fluid was subjected to electrofocusing in a 110-ml LKB electrofocusing column using the procedure described in the LKB Instruction Manual. Since a precipitate sometimes formed in the anode solution of sulfuric acid, it was found useful to have the anode as the lower electrode and if a precipitate formed during electrofocusing to collect samples from the top of the column by upward displacement with 50% sucrose. After 36–44 hr at 500 V and 2–5°, 1.5- to 2.0-ml fractions were collected. Groups of fractions with EF-1 activity were pooled and dialyzed against buffer A. Concentration of material either before or after electrofocusing was performed in an Amicon Diaflo ultrafiltration apparatus with a PM-30 membrane.

The material from electrofocusing was stable for several months when quick frozen and stored in liquid nitrogen. Enzyme stored at about 5° was stable for several days.

Phosphocellulose Chromatography. For phosphocellulose chromatography, which was used to separate multiple forms of EF-1 after electrofocusing, the sample was mixed with an equal volume of buffer A containing 2 mM $MgCl_2$ and 20% glycerol (AMG buffer), then chromatographed on a 1.2 × 5.1 cm column of phosphocellulose equilibrated with AMG buffer. Fractions of approximately 1.8 ml were collected.

Results

Multiple Forms. Figure 1 demonstrates the behavior of EF-1 on a Sepharose 6B column. There are two zones of activity, one of which peaks at fraction 50 and a second at fraction 80. The second zone elutes between hemoglobin and EF-2 (molecular weight near 100,000: Collins *et al.*, 1971b) and thus has a molecular weight of 80,000–90,000. This peak probably corresponds to the smaller of the two peaks of EF-1 from a Sephadex G-200 column reported previously (Schneir and Moldave, 1968), and is considered further below. By its position of elution the zone peaking around fraction 50 was estimated to be of mol wt 200,000 or greater. Schneir and Moldave (1968) were able to resolve two components in their larger molecular weight fraction by rechromatography. We also showed this by running the fraction on a sucrose

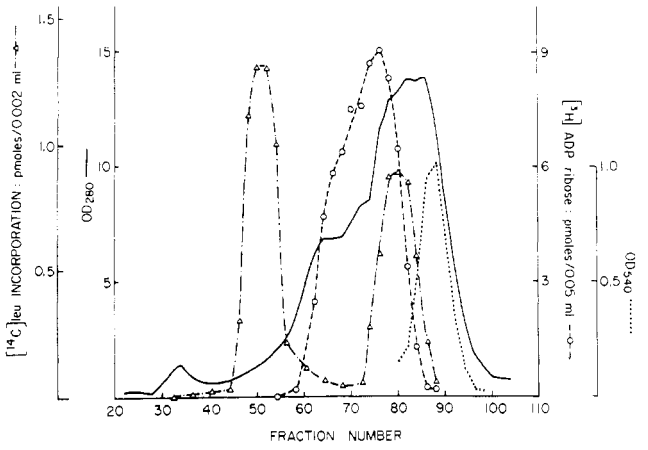

FIGURE 1: Gel filtration of EF-1 on Sepharose 6B. Fifty milliliters (3650 mg) of the dialyzed 40–70% $(NH_4)_2SO_4$ precipitate were used. The ultracentrifugation step was not used in this experiment, so that the flow rate was 5 ml/hr. EF-1 activity was determined by complementation with EF-2 for amino acid incorporation. EF-2 was determined by ADP ribose incorporation in the presence of diphtheria toxin. OD_{540} was used to determine the peak of hemoglobin.

gradient (Figure 2A). There were species of approximately 400,000 and 170,000 molecular weight. The first peak from the Sepharose 6B column was chromatographed on a hydroxylapatite column, from which two protein peaks were eluted. The single peak of EF-1 activity was associated with the later eluting, lower OD_{280} peak. The enzyme was then subjected to electrofocusing at pH 3–8. As shown in Figure 3, there are two peaks of EF-1 activity as measured either by leucine incorporation into polypeptide from Leu-tRNA or by the binding of Phe-tRNA to poly(U)-programed ribosomes. Both peaks also had ribosome-dependent GTPase activity (data not shown). Each of these peaks from electrofocusing was finally resolved into similar multiple forms by phosphocellulose chromatography (Figure 4) or on 5.2% polyacrylamide gels (Figure 5). Usually two or more protein bands were seen on the gels, with two zones of activity which were at the same place on the gels from each electrofocusing peak but which showed quantitative variation between experiments. The electrofocusing peak with the lower p*I* had more of the slower moving zone of activity, while the peak with the higher p*I* was enriched for the faster moving zone of activity. Each peak from electrofocusing showed a similar heterogeneous

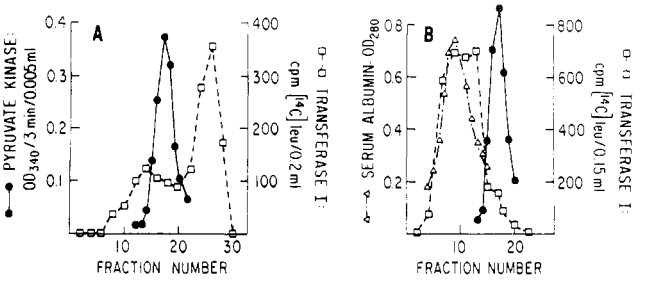

FIGURE 2: Sucrose gradient centrifugation of EF-1. (A) Sepharose 6B fraction. The sample contained 15,000 cpm of EF-1 activity in the [^{14}C]Leu-tRNA assay. Serum albumin was omitted. Centrifugation was for 50.4 hr at 22,400 rpm. (B) Electrofocusing. The sample contained 29,000 cpm of the second electrofocusing peak, that had been pooled and dialyzed against buffer A. Centrifugation was for 48.8 hr at 22,100 rpm.

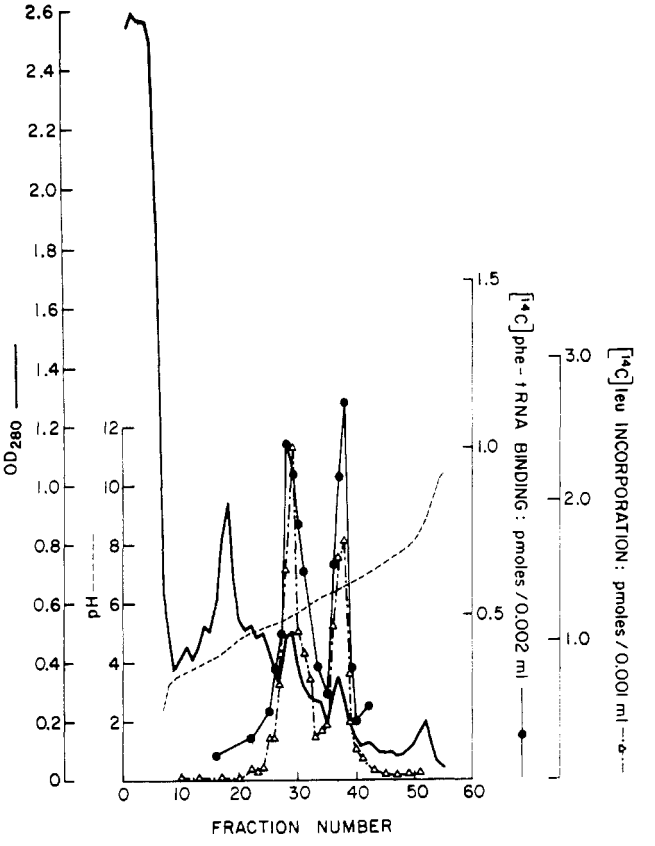

FIGURE 3: Electrofocusing of EF-1 at pH 3-8. A 13-ml (approximately 39 mg) sample from the hydroxylapatite column was prepared for electrofocusing as described in Methods. The run was terminated after 39 hr at 500 V.

pattern on sucrose gradients with components in the range of 60,000–170,000 molecular weight (Figure 2B). Since the existence of the multiple forms of activity could complicate discrimination of EF-1 from contaminants, the enzyme was not purified further. Although two peaks of activity were seen on electrofocusing columns, the isoelectric points for the two peaks were variable. In contrast, in purifications of EF-2 the p*I* has been reproducible (Raeburn *et al.*, 1971). The yield of EF-1 has been approximately 1%. The overall purification is usually 30- to 60-fold (Table I), but in individual preparations has been 100-fold and higher. The two peaks from electrofocusing had similar specific enzyme activity, were similarly inactivated by preincubation with GTP (*cf.* Table IV) and when run on sodium dodecyl sulfate–acrylamide gels, contained a main band of mol wt 53,000 plus variable minor bands.

Enzymic Activity. By an analysis similar to that done for EF-2 (Raeburn *et al.*, 1971), purified EF-1 is catalytic in amino acid incorporation, promoting the incorporation of up to 10 moles of amino acid from [^{14}C]Leu-tRNA per 100,000 g of EF-1 in 10 min at 37°, using polysomes containing endogenous mRNA and peptidyl-tRNA. An approximate value of 100,000 was used for the molecular weight based on the sucrose gradient analysis (Figure 2B). However, the purified enzyme is probably stoichiometric in binding [^{14}C]Phe-tRNA to ribosomes in the presence of poly(uridylic acid). Approximately 0.1–0.25 mole of Phe-tRNA was bound to ribosomes per 100,000 g of protein present. The inability of EF-1 to act catalytically in binding Phe-tRNA to ribosomes (*i.e.*, less than one Phe-tRNA bound to ribosomes per mole of enzyme)

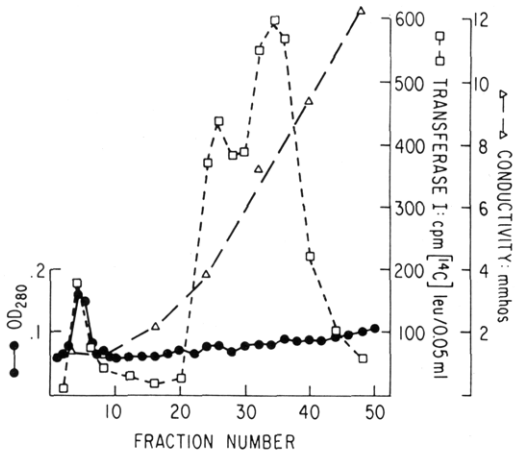

FIGURE 4: Chromatography of EF-1 from electrofocusing on phosphocellulose. The enzyme from the second peak of activity in Figure 3 (higher p*I*) was dialyzed against buffer A. A 1.2-ml sample containing 0.46 mg of protein was mixed with 1.2 ml of AMG buffer and applied to a 1.2 × 5.2 cm column of phosphocellulose equilibrated with AMG buffer. The column was stopped during sample addition to allow equilibration. (The small peak of activity at fraction 4 may be due to incomplete equilibration.) The column was developed as described in Methods. Both major peaks of incorporation activity were shown to have activity in binding Phe-tRNA to ribosomes. When the peak of activity with the lower p*I* in Figure 3 was similarly chromatographed, activity appeared in the same places on the column, but a greater percentage of the activity eluted at lower KCl concentrations.

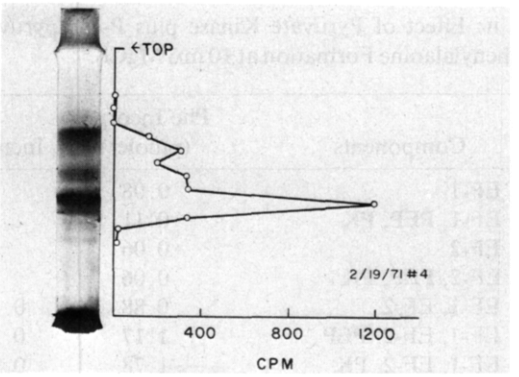

FIGURE 5: Elution of EF-1 from native polyacrylamide gels. Gels were run, sliced, and eluted as described in Methods. After standing in a refrigerator overnight, the fluid in the tubes was assayed for EF-1 by complementation with EF-2. To the gel shown approximately 24 μg of EF-1 protein from electrofocusing was applied. Greater than half the EF-1 activity applied to the gel was recovered.

may mean that EF-1 binds to the ribosome and is able to function again only when the other steps in the cycle of protein synthesis cause it to be displaced from the ribosome.

Purified EF-1 is active in directing polyphenylalanine formation in the presence of poly(U), ribosomes, and EF-2. At low concentrations of EF-1 in the assay, the curve of polyphenylalanine formed *vs.* EF-1 present was sigmoid, due at least partly to synthesis of short polyphenylalanine chains since the addition of carrier protein to the reaction increased the radioactivity precipitated on the filter. There still remained a slight sigmoid effect, similar to that seen earlier (Arlinghaus *et al.*, 1968). This result may be due to the necessity of EF-1 and EF-2 to act on the same ribosome for a chain to be formed and elongated. With constant amounts of the two elongation factors as the amount of ribosomes is increased, polyphenylalanine formation first increases and then declines. (For example, with 8 μg of EF-1 from the Sephrose 6B step and 5.5 μg of EF-2 from the phosphocellulose step (Raeburn *et al.*, 1971), incorporation with 0.02 or 0.28 mg of ribosome was half that observed with 0.10 mg of ribosome.) As more ribosomes are added, the probability increases that with less than saturating amounts of the elongation factors, molecules of the two factors will associate with different ribosomes and thus not cooperate for chain elongation. Previous work (Ibuki and Moldave, 1968; Skogerson and Moldave, 1968b) demonstrated that both EF-1 and EF-2 can bind to ribosomes in the absence of the other, if appropriate reagents are present.

A stimulation of activity in the poly(U)-directed incorporation assay with limiting amounts of EF-1 was observed in the presence of phosphoenolpyruvate plus pyruvate kinase at 10 mM $MgCl_2$ (Table II) and a smaller effect was observed with pyruvate kinase alone. A smaller stimulation (less than twofold) was also seen with the polysome assay at 5 mM $MgCl_2$. This suggested that EF-1 as isolated might contain GDP, as purified EF-Tu (*E. coli*) does (Miller and Weissbach, 1970). However, whereas pyruvate kinase plus phosphoenolpyruvate can stimulate EF-Tu-directed binding of Phe-tRNA to ribosomes by partially replacing EF-Ts (Weissbach *et al.*, 1970), no stimulation was seen in the binding reaction directed by rat liver EF-1. Phosphoenolpyruvate plus pyruvate kinase should prevent the buildup of GDP which might inhibit incorporation by forming an EF-2–GDP–ribosome complex (Skogerson and Moldave, 1968b). However, stimulation occurs even at saturation levels of EF-2 when EF-1 is limiting for incorporation. GDP can also labilize EF-1 even when aminoacyl-tRNA is present (Ibuki and Moldave, 1968), but the virtual absence of GDP cannot be the only reason for the stimulation since a half-maximal effect is seen when the P-enolpyruvate added is only one-third the GTP added (and thus one-third the amount of GDP that could be produced). The basis of the stimulation of incorporation is

TABLE I: Purification of EF-1.

Fraction	Vol (ml)	Protein (mg/ml)[a]	Total Act. (× 10^{-6})[b]	Purification -fold
1. Postmitochondrial fraction	340.0	76.2	121.4	1.0
2. pH 5 supernatant solution	295.0	48.4	58.1	0.9
3. 40–70% $(NH_4)_2SO_4$	65.5	94.7	35.4	1.2
4. Ultracentrifugation	63.5	97.3	34.9	1.2
5. Sepharose 6B, first peak (dialyzed)	169.0	1.28	7.5	7.3
6. Hydroxylapatite (dialyzed)	21.5	2.31	2.7	11.6
7. Electrofocusing, first peak (dialyzed)	6.0	0.10	0.15	52.8
8. Electrofocusing, second peak (dialyzed)	13.1	0.20	0.59	47.9

[a] Protein was determined by the method of Lowry *et al.* (1951) in steps 1–4 and by the method of Warburg and Christian (1942) in the later steps. [b] Activity units are cpm of [^{14}C]leucine incorporated in 10 min at 37°.

TABLE II: Effect of Pyruvate Kinase plus P-enolpyruvate on Polyphenylalanine Formation at 10 mM $MgCl_2$.[a]

Components	Phe Incorpn (pmoles)	Increase
1. EF-1	0.08	
2. EF-1, PEP, PK	0.11	
3. EF-2	0.06	
4. EF-2, PEP, PK	0.06	
5. EF-1, EF-2	0.88	0
6. EF-1, EF-2, PEP	1.17	0.29
7. EF-1, EF-2, PK	1.78	0.90
8. EF-1, EF-2, PEP, PK	3.43	2.55

[a] The assay contained the components for the poly(U) assay as listed in the Methods and where indicated 1.4 μg of EF-1 from the electrofocusing step, 4 μg of EF-2 from the phosphocellulose step (Raeburn *et al.*, 1971), 10 μg of pyruvate kinase (Sigma), and 2 mM phosphoenolpyruvate. PEP = phosphoenolpyruvate; PK = pyruvate kinase.

not known, but no evidence for a Ts-like factor in the liver system is available.

GTPase Activity. A GTPase activity that was dependent on ribosome and aminoacyl-tRNA was found in the purified reticulocyte enzyme (Lin *et al.*, 1969). As shown in Table III, the GTPase activity of liver EF-1 requires ribosomes and aminoacyl-tRNA. Comparing GTP hydrolyzed with Phe-tRNA bound to ribosomes (although the assay conditions are somewhat different), there are more than ten molecules of GTP split per Phe-tRNA bound by the same amount of enzyme. This phenomenon of uncoupled activity was also seen with a less purified enzyme (Felicetti and Lipmann, 1968). EF-1 alone (Table III, bottom line) showed an activity of approximately 5% that of the ribosome-dependent activity.

GTP and Enzyme Lability. The inactivation of EF-1 in the presence of GTP and prevention by aminoacyl-tRNA was first demonstrated for the amino acid incorporation activity (Ibuki and Moldave, 1968). The three activities of rat liver EF-1 (binding of Phe-tRNA to ribosomes, GTPase, and complementation with EF-2 for amino acid incorporation) show similar lability to preincubation with GTP and protection against this lability by aminoacyl-tRNA (Table IV).

TABLE III: Ribosome-Dependent GTPase of EF-1.[a]

Omissions	[γ-^{32}P]GTP Hydrolyzed (pmoles)
None	110.7
Ribosomes	16.6
EF-1	<0.1
Aminoacyl-tRNA	<0.1
Aminoacyl-tRNA, ribosomes	6.1

[a] The assay conditions are given in Methods. In this experiment 9 μg of EF-1 from electrofocusing and 93 μg of ribosomes, free of endogenous mRNA and peptidyl-tRNA, were used.

TABLE IV: Effect of GTP on EF-1 Stability.[a]

Preincubn Components	% Act. Remaining: Incorpn Act.	GTPase	Phe-tRNA Binding
EF-1	100	100	100
EF-1, GTP	59	47	36
EF-1, GTP, aminoacyl-tRNA	93	91	96

[a] EF-1 from electrofocusing was incubated with the indicated preincubation components in the salts mixtures for the various assays. After 10–30 min at 37°, the other components for the assay were added and incubation was carried out as usual.

Similar results were seen with the rabbit reticulocyte enzyme (Lin *et al.*, 1969). The parallel lability of the three activities suggest they are all due to the same molecule. Figure 3 also indicates this, since all three activities are found in the same peaks after electrofocusing.

Inhibition of Binding Activity by EF-2. In order to follow the activity of EF-1 in binding [^{14}C]Phe-tRNA to ribosomes during enzyme purification, it was necessary to add GDPCP to the reaction mixture to prevent amino acid polymerization in cases where EF-2 activity overlapped EF-1 activity, such as seen in Figure 1. Much less binding activity was found than expected on the basis of the incorporation assay. To determine if there was an inhibition due to the presence of EF-2, highly purified preparations of both enzymes were used. As shown in Table V, EF-2 inhibits the binding activity due to EF-1. It also appears to inhibit non-enzymic binding. Lines 6 and 7 indicate that EF-2 can displace [^{14}C]Phe-tRNA previously bound by EF-1.

Second Form of Elongation Factor 1 from Sepharose 6B. As shown in Figure 1, there is a second peak of EF-1 activity with molecular weight near 80,000 which may correspond to the smallest form of EF-1 (estimated molecular weight of

TABLE V: Inhibition by EF-2 of EF-1 Directed Binding.[a]

Conditions	Incubn (min)	[^{14}C]Phe-tRNA Bound (pmole)
1. No enzymes	20	0.30
2. EF-1	20	0.79
3. EF-2	20	0.06
4. EF-2 plus EF-1	20	0.10
5. EF-1, then EF-2 added at end of incubation	20	0.76
6. EF-1	10	0.61
7. EF-1, add EF-2 at 10 min	20	0.24

[a] The conditions for the binding assay are given in Methods. In this experiment, 1.8 μg of EF-2 from the phosphocellulose step (Raeburn *et al.*, 1971) and 1.5 μg of EF-1 from an electrofocusing step were used. GDPCP (0.5 mM) replaced GTP. Incubation was carried out for the indicated times at 37°.

100,000) seen by Schneir and Moldave (1968) on a Sephadex G-200 column. In different preparations this peak accounted for 10–50% of the the EF-1 activity. It was difficult to determine with fractions from the Sepharose column if this peak had activity in binding Phe-tRNA to ribosomes due to interference by EF-2 which overlaps this peak (Figure 1 and Table III). To separate the activity from EF-2, the activity was chromatographed on hydroxylapatite and on both DEAE-cellulose and phosphocellulose, and in all cases recovery of activity was very low. (Mixing experiments with various fractions did not indicate that two components had been separated.) When 2 mM $MgCl_2$ and 20% glycerol were added to stabilize the enzyme, recovery was improved. The activity, designated EF-1′ (or transferase I′), was partially separated from EF-2 on hydroxylapatite and nearly completely on a phosphocellulose column (Figure 6). Polyacrylamide gels showed two to five bands for the EF-1′ from the last step and several bands on sodium dodecyl sulfate. At this stage in one experiment there was a 27-fold purification but only a 2% recovery in the phosphocellulose step compared to the Sepharose step. (Further purification on DEAE-cellulose in AMG buffer with a KCl gradient yielded two peaks of activity one of which gave only one band on polyacrylamide gels.) EF-1′ from the phosphocellulose step was active in complementing EF-2 in the assay using polysomes (Figure 6) and in the assay for polyphenylalanine formation, and promoted the binding of Phe-tRNA to ribosomes. However, if the EF-1′ from the phosphocellulose step is around 25% pure, one Phe-tRNA is bound per approximately 25 EF-1′ molecules present. Perhaps another factor is required for the binding or EF-1′ may be unstable and largely denatured. EF-1′ does not appear to be the factor equivalent to EF-1 in mitochondria since an EF-1′ fraction from Sepharose 6B did not replace EF-T (*E. coli*) in an *E. coli* cell-free system (Bernardi and Leder, 1970). It is probably not an initiation factor since it cannot replace factor M_1 or M_2 in the initiation system from rabbit reticulocytes (Prichard *et al.*, 1970; Crystal *et al.*, 1971).

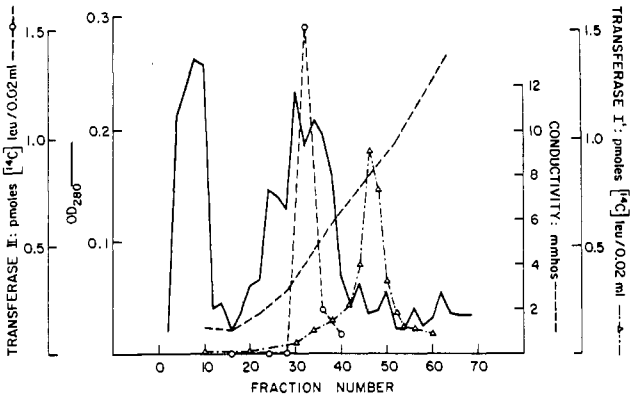

FIGURE 6: Chromatography of EF-1′ on phosphocellulose in the presence of 2 mM $MgCl_2$ and 20% glycerol. The second peak of EF-1 from a Sepharose 6B column, such as in Figure 1, was pooled, dialyzed against 0.01 M potassium phosphate (pH 7.0), 1 mM dithiothreitol, 2 mM $MgCl_2$, 20% glycerol, and chromatographed on a column (30-ml bed volume) of hydroxylapatite equilibrated with the same buffer. The column was developed with a 600-ml linear gradient of 0.01–0.5 M potassium phosphate (pH 7.0), containing the other components as above. The peak of EF-1′ activity was pooled and dialyzed *vs.* AMG buffer. Eighty-three milliliters (29 mg) was chromatographed on a 3.1 × 3.7 cm column of phosphocellulose equilibrated with AMG buffer. The column was developed with a 600-ml linear gradient of 0–0.5 M KCl in AMG buffer. Fractions (10 ml) were collected at a flow rate of 120 ml/hr.

Discussion

Although EF-1 was resolved from EF-2 several years ago (Gasior and Moldave, 1965) and purification procedures for EF-2 have appeared (Galasinski and Moldave, 1969; Raeburn *et al.*, 1971; Honjo *et al.*, 1971), no extensive purification for EF-1 has previously been published. The overall purification in this report comparing the enzyme from isoelectric focusing to the postmitochondrial supernatant fraction is approximately 50-fold. The difficulty in obtaining homogeneous material from rat liver may be due to the existence of multiple forms as seen on Sepharose 6B columns, electrofocusing, phosphocellulose columns, and in polyacrylamide gels. Previously multiple forms had only been shown on Sephadex G-200 columns (Schneir and Moldave, 1968). The significance of the various multiple forms is not known. Two peaks of activity on Sepharose 6B are seen in all preparations although the relative proportions vary. In the further treatment of the Sepharose fraction larger than EF-2, there is a shift to smaller sizes of the active components, as shown by sucrose density gradient centrifugation. The 400,000 molecular weight (14S) component may be related to the 19S particle reported earlier (Shelton *et al.*, 1970). There are regularly two forms of enzyme after isoelectric focusing but the p*I* vary. The peaks from electrofocusing show at least two peaks of activity that absorb to a phosphocellulose column. Neither the two forms from Sepharose 6B (Figure 1) nor the two forms from electrofocusing (Figure 5) are generated by the pH 5 step or the ammonium sulfate fractionation, since they appear when these steps are omitted (data not shown). In addition the two forms from Sepharose do not appear to be in rapid equilibrium with each other under the conditions of the chromatography since they rechromatograph nearly completely at the same place on the column. The larger molecular weight component(s) from Sepharose after purification through electrofocusing appears to be more active by a factor of approximately 10 in binding Phe-tRNA to ribosomes than the smaller molecular weight component purified through phosphocellulose, if it is assumed that EF-1′ is 25% pure. The latter (EF-1′) might be a less active and less stable product of the proteolysis of the former. EF-1′ does not seem to be an initiation factor or a mitochondrial factor corresponding to EF-1, since it cannot replace bacterial factor EF-T as eucaryotic mitochondrial factors do.

Until recently, data such as shown in Table IV have been the only evidence, and admittedly indirect, of the formation of an EF-1–GTP–aminoacyl-tRNA complex (Ibuki and Moldave, 1968; Lin *et al.*, 1969). Ternary complexes have been directly demonstrated in bacterial systems for EF-T factor (specifically EF-Tu)–GTP–aminoacyl-tRNA (reviewed by Lucas-Lenard and Lipmann, 1971). We have been unable to demonstrate a ternary complex using a more purified enzyme, with either a column technique such as Rao and Moldave (1967) or by Millipore filtration. However, a binary complex formed with partially purified EF-1 from calf brain and GTP is stable enough to be detected by Millipore filtration (Moon and Weissbach, 1972). The ability of [^{14}C]Phe-tRNA to release the complex from the filter indicates that a ternary complex is probably formed after the binary complex. These authors have recently obtained evidence that there are two forms of EF-1 in calf brain extracts, one of which $EF\text{-}1_A$ binds GTP and a second, smaller form $EF\text{-}1_B$ which binds GDP. $EF\text{-}1_A$–GTP reacts with Phe-tRNA to form a Phe-tRNA–$EF\text{-}1_B$–GTP complex (Moon *et al.*, 1972).

The interference by EF-2 in EF-1 directed binding (Table V) may indicate that EF-2 binds at a site overlapping the site for Phe-tRNA binding. Furthermore, EF-2 displaces Phe-tRNA already bound. It is unlikely that EF-2 has translocated the Phe-tRNA to the peptidyl site (at which site it might be loosely bound and dissociate), since translocation does not occur in the presence of GDPCP (Skogerson and Moldave, 1968a). An overlap of sites on the bacterial ribosome is indicated by the inhibition by certain antibiotics (thiopeptin and siomycin) of partial reactions of both EF-T and EF-G (Kinoshita *et al.*, 1971) and by the ability to greatly reduce the ribosome-dependent GTPase activities of both EF-T and EF-G factors by the removal of one ribosomal protein without damaging other ribosomal functions (Hamel and Nakamoto, 1971; Hamel *et al.*, 1972; Weissbach *et al.*, 1972). Recently several papers have appeared showing that EF-G inhibits the binding of aminoacyl-tRNA to ribosomes (Cabrer *et al.*, 1972; Miller, 1972; Richman and Bodley, 1972; Richter, 1972). Indeed since EF-1 binds aminoacyl-tRNA to the acceptor site while EF-2 moves the peptidyl-tRNA from the acceptor to the donor site, it is not surprising that some overlap may occur.

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