# PROTEIN SYNTHESIS ELONGATION FACTOR 1 FROM RAT LIVER: A ZINC METALLOENZYME

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Highly purified elongation factor 1 (light form, $EF1_L$) from rat liver contains zinc as determined by atomic absorption spectrophotometry. Analysis has been performed on the most active protein fraction from DEAE-Sephadex chromatography (estimated purity: 90%) and on the main band obtained from this fraction by polyacrylamide gel electrophoresis. The data are consistent with a stoichiometry of approximately one g-atom of zinc per 54,000 daltons of $EF1_L$ protein. A functional role for $Zn^{2+}$ is suggested by the fact that 0.3 mM 1,10-phenanthroline completely abolishes GTP binding by $EF1_L$ (measured by the nitrocellulose filter retention assay), while the isomeric non-chelator 1,7-phenanthroline has no effect. This inhibition can be overcome by the addition of excess zinc ion.

Tightly bound zinc ions play important structural and functional roles in a number of enzymes of nucleotide and nucleic acid metabolism. Notable examples include aspartate transcarbamylase (1), DNA polymerase I (2) and RNA polymerase (3), all from Escherichia coli, and the reverse transcriptases of avian myeloblastosis virus (4), and murine, simian, feline, and RD-114 RNA tumor viruses (5). In this laboratory we have begun a study of the homologous protein synthesis elongation factors EF-T (E. coli) and EF1 (rat liver)**. Elongation factor 1, a soluble protein, catalyzes the binding of aminoacyl-tRNA to the mRNA-ribosome A site through the formation of an aminoacyl-tRNA•EF1•GTP ternary complex. Hydrolysis of GTP releases EF1•GDP from the ribosomal site (6). The next steps in protein synthesis, formation of the peptide bond and translocation of the newly lengthened peptidyl-tRNA from the A site to the P site are cata-

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**Abbreviations used: $EF1_L$ -- elongation factor 1 (light form), $EF1_H$ -- elongation factor 1 (heavy form).

lyzed by peptidyl transferase (part of the large ribosomal subunit) and another soluble protein, EF2 (rat liver) or EF-G (E. coli), respectively. The prokaryotic factor EF-T consists of two entities, EF-Tu and EF-Ts, which have been crystallized (7). Previously EF1 from various animal sources (8,9) has been difficult to purify, partly because of its association with phospholipids. Its isolation resulted in aggregates ranging in size from M.W. ≥200,000 ($EF1_H$) to the light form ($EF1_L$), M.W. 50-60,000, as determined by disc gel electrophoresis and sucrose gradient centrifugation. Using these same criteria, EF1 from rabbit reticulocytes has been purified to apparent homogeneity and comprises three identical subunits of 62,000 daltons each (10). $EF1_L$ from calf brain has been shown to be five times more efficient in forming the ternary complex than $EF1_H$ (11), suggesting that the former is the functionally active species. Unpublished reports of the presence of zinc in EF-Tu (12) prompted us to examine our $EF1_L$ preparations for the presence of this metal.

## MATERIALS AND METHODS

The procedure used in the purification of EF1 was that of Collins et al. (8) with the following modifications: A combined column of Sepharose 4B overlayed with Sephadex G-200 (2:1 vol/vol) was used instead of Sepharose 6B gel filtration. DEAE-Sephadex A-50 chromatorgraphy (1.5 x 40 cm column) followed the hydroxylapatite step and used a linear 0.1-0.5 M KCl gradient in 20 mM Tris-HCl (pH 7.5), 1 mM DTT and 0.1 mM EDTA. The final column eluted $EF1_L$ at 0.4 M KCl. (See Table I.)

Unfractionated E. coli tRNA was prepared and aminoacylated with [$^{14}$C]-phe according to (13).

The assay for EF1 activity was based on the ability of EF1 to bind GTP, forming a complex which is retained on nitrocellulose filters (14). The reaction of the EF1•GTP complex with phe-tRNA to form a phe-tRNA•EF1•GTP ternary complex that is not retained on nitrocellulose filters was routinely performed as described (14) with the modification that phe-tRNA was incubated with EF1•GTP for only 30 sec. at 0°. All radioactivity determinations were made using a Picker scintillation counter and a water-miscible scintillation fluid (15).

Sodium-dodecyl-sulfate gel electrophoresis (16) and sucrose gradient centrifugation, 5-20%, (17) were used to determine the molecular weight of $EF1_L$ using known standard proteins as markers. Both methods agreed closely with a M.W. of 54,000 ± 4,000. The EF1 preparations following either hydroxylapatite or DEAE-Sephadex A-50 chromatography were analyzed using polyacrylamide gel electrophoresis (18) and determined to be approximately 60% and 90% pure $EF1_L$, respectively, by densitometric analysis. To assay for EF-1 activity, the gels were sliced and treated as described in (8). GTP-binding activity was eluted from two major bands with approximately 60% recovery of EF1 activity (Figure 1).

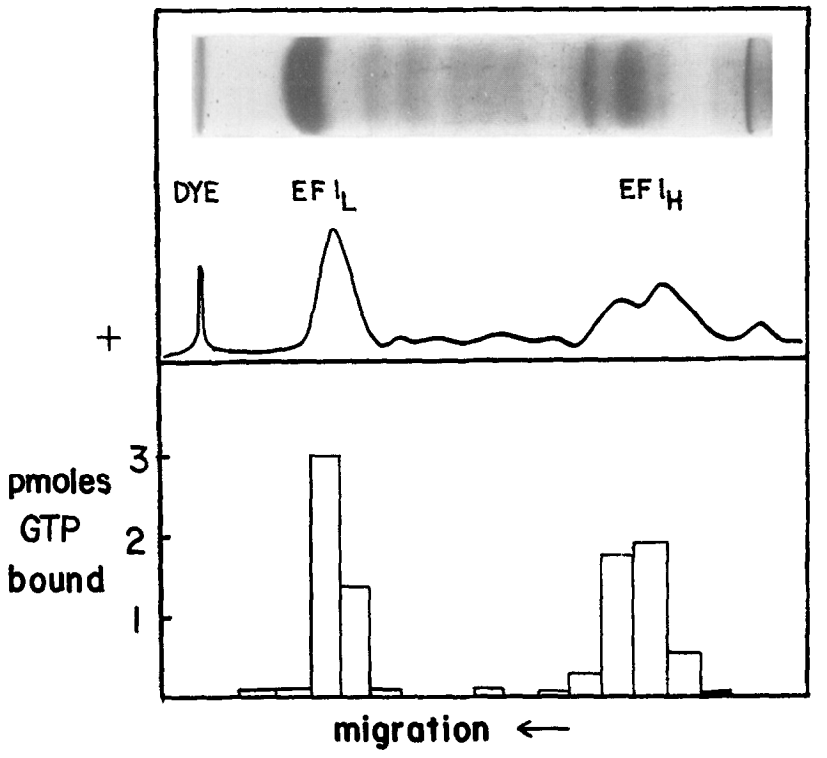


Figure 1. Analysis of EF1 Preparation by Polyacrylamide Gel Electrophoresis.

Top frame: Photograph of gel stained with Coomassie Blue and densitometer tracing of the same sample (from hydroxylapatite). Bottom frame: EF1 activity profile derived from slices of a gel identical to that depicted above. Protein was extracted from the slices and assayed as described in MATERIALS AND METHODS.

To assay for $Zn^{2+}$ all glassware and standards were treated as described by Thiers (19) to avoid metal ion contamination. The gel slices corresponding to $EF1_L$ were digested with 2.0 ml concentrated $HCl/HNO_3$ (3:1) and if necessary further oxidized with 1.0 ml hydrogen peroxide (BDH, ultra-pure). The residue was dissolved in 1.0 ml 1% $HNO_3$ to give a clear solution. In cases where analyses were performed directly on free enzyme in solution, the sample was digested with 8 N $HNO_3$ to evaporation and the residue dissolved as above. All assays were done using a Perkin-Elmer 305A atomic absorption spectrophotometer with the flame attachment and the resonance line at 2138 A (20).

In routine analyses protein concentration was determined by the method of Warburg and Christian (21). Absorbances at 280 and 260 nm were measured on a Zeiss PMQ II spectrophotometer. When highly pure enzyme was used protein concentration was also determined by the Lowry method (22) with crystalline bovine serum albumin as standard. The two methods agreed within 10%.

[$^{14}$C]-phenylalanine and [ $^{3}$H]-GTP were obtained from New England Nuclear; ATP, GTP, pyruvate kinase and phosphoenolpyruvate were from Sigma; hydroxylapatite (hypatite C) was from Clarkson Chemical Co.; Sephadex G-200, Sepharose

TABLE I. Purification of $EF1_L$ from Rat Liver

Figures in the table refer to a preparation starting from 340 g (wet weight) of frozen liver. One unit of EF1 activity binds one pmol GTP per minute per milligram protein in the standard assay at 37°. Yields given in the table reflect the selection of the $EF1_H$ fractions after gel filtration and hydroxylapatite chromatography and the $EF1_L$ fractions after DEAE-Sephadex chromatography.

| Fraction | Vol. (ml) | Protein (mg) | Protein conc. (mg/ml) | Specific activity (units/mg) | Yield (%) |
|---|---|---|---|---|---|
| SN-100[a] | 500 | 28,000 | 56 | 4.8 | 100 |
| Ammonium sulfate (35-70%)[b] | 150 | 9,750 | 65 | 11.4 | 83 |
| Gel filtration | 40 | 600 | 15 | 32.0 | 14 |
| Hydroxylapatite | 30 | 75 | 2.5 | 133 | 7.4 |
| DEAE-Sephadex | 10 | 1.5 | 0.15 | 780 | 0.9 |

[a] post-microsomal fraction

[b] dialyzed vs. 50 mM Tris-HCl (pH 7.2) and 1 mM DTT

4B and DEAE-Sephadex A-50 were from Pharmacia; nitrocellulose filters were from the Millipore Co.; 1,10-phenanthroline was purchased from K & K Laboratories, and 1,7- and 4,7-phenanthroline were purchased from the G.F. Smith Co. The latter two compounds were once-recrystallized from ethanol/water mixtures before use. All other chemicals were reagent grade.

## RESULTS

### Metal Content of $EF1_L$

We performed atomic absorption analyses for $Zn^{2+}$ on gel slices containing the $EF1_L$ protein band from which enzyme activity could be eluted (Figure 1) as well as the free enzyme solution after DEAE-Sephadex chromatography. The rat liver $EF1_L$ contained 1.3 $\pm$ 0.3 (SD) g-atom $Zn^{2+}$/mole of enzyme of molecular weight 54,000. As shown in Table II, in each case where the enzyme sample was halved there was good correlation in the net ppm $Zn^{2+}$ observed. The fact that the $Zn^{2+}$/protein ratio increased from 0.4 µg $Zn^{2+}$/mg protein at the hydroxyla-

TABLE II. Zinc Content of $EF1_L$ from Rat Liver

The mean value for zinc content of $EF1_L$ is 1.3 ± 0.3 g-atom per mole protein (mol. wt. 54,000). Preparation A is from hydroxylapatite, estimated 60% pure $EF1_L$ by densitometric analysis of polyacrylamide gels; preparation B is from DEAE-Sephadex A-50, 90% pure. All analyses were done with gel slices except where noted. The blank consisted of a protein-free gel slice except in the case of free enzyme analyses which had a blank containing 50 µg bovine serum albumin.

| Preparation No. | EF1 Present (µg $EF1_L$) | Observed Zinc[a] (ppm) | Zinc Content (g-atom mole) |
|---|---|---|---|
| A-1 | 65 | 0.115 (0.050) | 1.46 |
| A-2 | 60 | 0.083 (0.045) | 1.14 |
| | 30 | 0.040 (0.045) | 1.10 |
| A-3 | 36 | 0.068 (0.072) | 1.56 |
| | 18 | 0.036 (0.072) | 1.65 |
| A-4[b] | 60 | 0.073 (0.042) | 1.01 |
| B-1 | 77 | 0.123 (0.052) | 1.32 |
| B-2[b] | 36 | 0.042 (0.055) | 0.96 |

[a] Values in the table are the net ppm zinc observed; the corresponding blanks are given in parentheses.

[b] In these cases $EF1_L$ solutions were hydrolyzed directly in 8 N $HNO_3$ with gentle heating.

patite stage of purification to nearly 1.0 µg $Zn^{2+}$/mg protein at the final purification stage indicates $Zn^{2+}$ is an integral part of the enzyme.

Effect of Chelators on $EF1_L$•GTP Binding Activity

We found complete inhibition of EF1•GTP activity (i.e. no binary complex was retained on nitrocellulose filters) when 1,10-phenanthroline was present in the reaction mixture at a concentration equal to or greater than $3 \times 10^{-4}$ M. Lower concentrations of 1,10-phenanthroline were less effective in inhibiting activity (see Table III). The activity was restored to 90% of the control with $10^{-4}$ M $Zn^{2+}$. The non-chelating isomers, 1,7-phenanthroline and 4,7-phenanthroline, had no effect on the GTP-binding activity. The effect of 1,10-phenan-

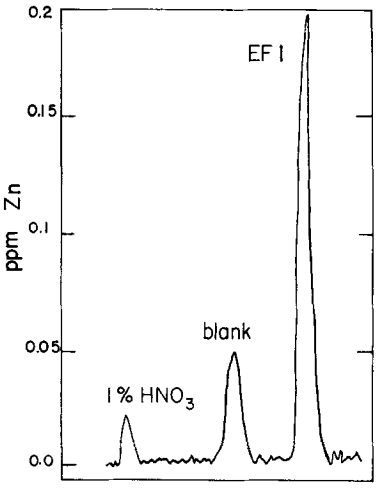


Figure 2. Recorder Chart Tracing from Atomic Absorption Analysis of $EF1_L$.

The ordinate calibration comes from $Zn^{2+}$ standard solutions run on the same day. The sample consisted of a slice from a polyacrylamide electrophoresis gel containing the $EF1_L$ band (see Figure 1); the blank corresponds to an equivalent slice of a protein-free gel. Both slices were digested as described in the text and dissolved in 1% $HNO_3$ for analysis.

throline cannot be attributed to its possible removal of $Mg^{2+}$, since $Mg^{2+}$ is present in large excess (10 mM) in the reaction mixture and it is known that 1,10-phenanthroline complexes much more strongly with $Zn^{2+}$ than with $Mg^{2+}$ (23).

EDTA inhibited much less effectively than 1,10-phenanthroline. A comparatively high concentration of EDTA (1 mM) reduced GTP-binding activity by only 50%. Since the final buffer during isolation contained $10^{-4}$ M EDTA, inhibition by chelating agents must represent removal of tightly bound $Zn^{2+}$ ions. As with the inhibition by 1,10-phenanthroline, addition of $10^{-4}$ M $Zn^{2+}$ to EDTA-inhibited $EF1_L$ restored enzyme activity.

## DISCUSSION

Because of the relatively small absolute amounts of $Zn^{2+}$ ( 0.1 ppm) in $EF1_L$ preparations and the rather considerable chances of adventitious $Zn^{2+}$ contamination, analytical data alone would not suffice to identify this protein with certainty as a zinc metalloenzyme. Thus, although our atomic absorption

TABLE III. Effect of Chelating and Non-chelating Isomers on EF1-Nucleotide Binding Activity

Each assay (0.25 ml total volume) consisted of 25 µl enzyme solution which contained 80 µg EF1, 25 µl reagent (incubated with EF1 for 45 minutes prior to reaction), and GTP-binding mixture of 150 µl, containing 0.10 M Tris-HCl (pH 7.4), 0.10 M $NH_4Cl$, 0.02 M $MgCl_2$, $3.75 \times 10^{-3}$ M phosphoenolpyruvate, 10 µg pyruvate kinase and $2.5 \times 10^{-6}$ M [$^3$H] - GTP.

| System | pmol GTP Bound | % Inhibition |
|---|---|---|
| Control | 16.7 | 0 |
| + 1.0 mM 1,10-phenanthroline[a] | 0.0 | 100 |
| + 0.3 mM 1,10-phenanthroline | 0.0 | 100 |
| + 0.1 mM 1,10-phenanthroline | 1.7 | 90 |
| + 0.01 mM 1,10-phenanthroline | 13.4 | 20 |
| + 10.0 mM EDTA | 5.0 | 70 |
| + 1.0 mM EDTA | 8.3 | 50 |
| +0.1 mM 1,10-phenanthroline + 0.1 mM $Zn^{2+}$[b] | 15.0 | 10 |
| + 1.0 mM EDTA + 0.1 mM $Zn^{2+}$ | 15.9 | 5 |
| + 1.0 mM 1,7-phenanthroline | 17.0 | 0 |
| + 1.0 mM 4,7-phenanthroline | 16.7 | 0 |

[a]The phenanthroline isomers were added from stock solutions containing 10% methanol. A methanol blank (0.5 pmol GTP) was subtracted from the reported values.

[b]25 µl of 1 mM $ZnCl_2$ was added to the reaction mixture for an additional 3 min. incubation at 37° before terminating the reaction.

analyses (Table II) consistently showed the presence of approximately 1.0 g-atom $Zn^{2+}$/mole $EF1_L$, the demonstration of inhibition of GTP-binding by 1,10-phenanthroline or EDTA (Table III) represents essential supporting evidence. The absence of inhibition by the non-chelating phenanthroline isomers provides an important control (4).

Further experiments are required to specify the precise role of $Zn^{2+}$ in $EF1_L$ and to establish the stoichiometry with certitude. The $Zn^{2+}$ may merely aid in

maintaining the native protein conformation as in E. coli aspartate transcarbamylase (1), so that its removal causes partial denaturation with consequent loss of GTP-binding activity. A conformation-sensitive probe such as circular dichroism might provide a test of this hypothesis. In any event, removal of the $Zn^{2+}$ must not cause too drastic an alteration in structure, since replacement leads to almost full recovery of activity. Preliminary experiments indicate that $Zn^{2+}$ also influences the state of aggregation of EF1, removal of $Zn^{2+}$ favoring the conversion of $EF1_H$ to $EF1_L$, and vice versa. A similar involvement of $Zn^{2+}$ in a subunit aggregation/disaggregation process has recently been reported for mouse nerve growth factor (24). We have thus far been unsuccessful in attempts to substitute $Co^{2+}$ for $Zn^{2+}$ in reactivation experiments.

Demonstration of a functional -- as opposed to merely structural -- role for $Zn^{2+}$ in EF1 poses considerable difficulties because of the complexity of the system in which catalysis occurs, viz, an aggregate of 80S ribosome, mRNA, peptidyl-tRNA, aminoacyl-tRNA, $EF1_L$, and GTP. A presumptive role for the metal ion would be to assist phosphoryl transfer from GTP to $H_2O$ (or perhaps first to a ribosomal protein (25-27)). Magnetic resonance techniques might allow the exploration of this possibility (28).

The identification of rat liver $EF1_L$ as a zinc metalloenzyme has the immediate practical consequence that future experiments with this protein should be performed under conditions of well-defined metal ion concentration. Exposure to chelating agents such as EDTA may be harmful (as, for example, in the work of Drews et al. (6,29)) and deliberate addition of 0.1 mM $Zn^{2+}$ to buffers in the latter stages of purification is probably advisable in order to to maintain maximum activity, analogous to the case of T7 RNA polymerase (30). EF1 preparations from other sources (calf brain (14), rabbit reticulocyte (10), wheat germ (31,32), etc.) should be examined for the presence and possible functional role of zinc.

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