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Two chromatographically separable forms of *Escherichia coli* elongation factor Tu

[DEAE-Sephadex/GDP binding/poly(Phe) synthesis/gene products/ribosomal binding]

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ABSTRACT **Two forms of the elongation factor Tu from *Escherichia coli* have been separated by chromatography on DEAE-Sephadex A50. Obvious chromatographic artifacts have been ruled out by investigation of the elution profile of GDP (a component of the column buffer as well as a ligand of Tu) and by rechromatography of the two components, either separately to give the component peaks or together to give a double peak. The two components have been confirmed as Tu by the poly(uridylic)-dependent polyphenylalanine synthesis and by the distribution of the Tu protein as quantitated from sodium dodecyl sulfate/polyacrylamide gel electrophoresis. Complexes with the elongation factors Ts and G have also been ruled out by activity profiles and by quantitation of the protein distribution, again on gels. The distribution of the two forms between ribosomal and supernatant fractions has been examined: one is bound preferentially to the ribosomal fraction and the other is found in the supernatant fraction. The possible significance of this is discussed.**

Multiple roles of the bacterial elongation factor EF-Tu have been invoked for various reasons. In addition to its elongation role, the factor is a component of the RNA bacteriophage Qβ replicase (1). It has been proposed as an element in a system for regulating ribosomal RNA transcription (2, 3). Its structural gene has been located on two distinct and widely separated loci of the chromosome (4). Its presence has also been claimed in two distinct compartments in the bacterial cell—namely, in the cytosol and in the periplasmic space (5, 6). In spite of all of these observations, there has never been an indication of heterogeneity of the EF-Tu molecule. We have observed such a heterogeneity on DEAE-Sephadex chromatography and have published a preliminary report of our findings (7). Because of the surprising and important nature of this discovery, extensive controls are necessary in order to establish its credibility. These controls are the subject of this communication.

MATERIALS AND METHODS

Escherichia coli were grown as described (8), harvested in midlogarithmic phase, frozen, and stored in liquid nitrogen. The factors were then isolated by the standard procedure of Arai *et al.* (9). The only important modification of the procedure was the breakage of the cells. Cells (200 g) were suspended in 60 ml of extraction buffer [20 mM Tris·HCl, pH 7.4 at 0°/10 mM $Mg(OAc)_2$,/20 mM NH_4Cl/5 mM 2-mercaptoethanol], and DNase (0.1 mg) (Worthington) was added. The cells were then broken in a French pressure cell (Aminco, Silver Spring, Md) at 8000–12,000 psi (56–84 MPa) (10). The ruptured cells were mixed with 300 ml of extraction buffer and centrifuged at 18,000 rpm at 0° in a SS34 Sorvall rotor for 40 min. The supernatant fluid was removed and spun in a Beckman Ti35 rotor at 35,000 rpm for 4 hr at 0°. The top two-thirds of the supernatant liquid was collected (S100) and the fraction was further treated as described by Arai *et al.* (9).

Column Chromatography. The ammonium sulfate fractions were chromatographed on DEAE-Sephadex A50 (Pharmacia) columns as described by Arai *et al.* (9) (details are given in the individual experiments). The solutions containing EF-Tu1 or EF-Tu2 activity from the DEAE-Sephadex A50 columns were concentrated by precipitation at 60% ammonium sulfate saturation. The precipitate was dissolved and dialyzed against 20 mM Tris·HCl buffer (pH 7.8 at 0°) containing 10 mM $Mg(OAc)_2$, 10 μM GDP, 0.1 M KCl, and 5 mM 2-mercaptoethanol and passed through a Sephadex G-100 column (1.6 × 45 cm) equilibrated with the same buffer and eluted at a flow rate of 16 ml/hr; 4-ml fractions were collected.

Assays for the Elongation Factors. The assays for EF-Tu and EF-Ts were essentially as described (9). The assay for EF-Tu contained the following reagents in a volume of 100 μl: 10 mM Tris·HCl (pH 7.4 at 0°), 10 mM $MgCl_2$, 50 mM NH_4Cl, 0.25 μM [^{3}H]GDP (115 Ci/mmol, The Radiochemical Centre, Amersham), and 1 mM dithiothreitol. The amount of EF-Tu was determined by the extent of [^{3}H]GDP binding after 30 min of incubation at 0°. The reaction was terminated by the addition of 2 ml of the above buffer (without GDP and dithiothreitol) at 0° and filtered through a nitrocellulose membrane filter (0.45 μm pore size, Millipore). The filter was washed five times with 2-ml portions of the same buffer, dried, and assayed for radioactivity in a liquid scintillation spectrometer.

The assay for EF-Ts was based on the fact that the factor catalyzes the exchange reaction of [^{3}H]GDP with EF-Tu–GDP. Binding was therefore measured as described above but with only a 30-sec incubation at 0°.

The assay for EF-G was carried out as ribosome-dependent GTPase activity (11). The standard solution (20 μl) contained 50 mM Tris·HCl (pH 7.4 at 0°), 160 mM NH_4Cl, 10 mM $MgCl_2$, 12 μg of ribosomes washed as described (10), and 0.45 mM [^{3}H]GTP (2 Ci/mmol, the Radiochemical Centre). The reaction was started by the addition of 1 μl of [^{3}H]GTP. After a 10-min incubation at 30°, the reaction was stopped by applying an aliquot (5 μl) onto a polyethyleneimine-cellulose thin-layer plate. Unlabeled GDP and GTP were also applied as markers. The development was as described (12). After visualization of the markers under short-wavelength UV light, the spots corresponding to GTP and GDP were cut out and the radioactivity was assayed. The results are expressed as nmol of GTP hydrolyzed.

The poly(Phe) synthesis assay was as described (10). The 100-μl reaction mixture contained: 50 mM Tris·HCl (pH 7.4

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Abbreviations: EF-Tu, elongation factor Tu; EF-Ts, elongation factor Ts; EF-G, elongation factor G; $NaDodSO_4$, sodium dodecyl sulfate.

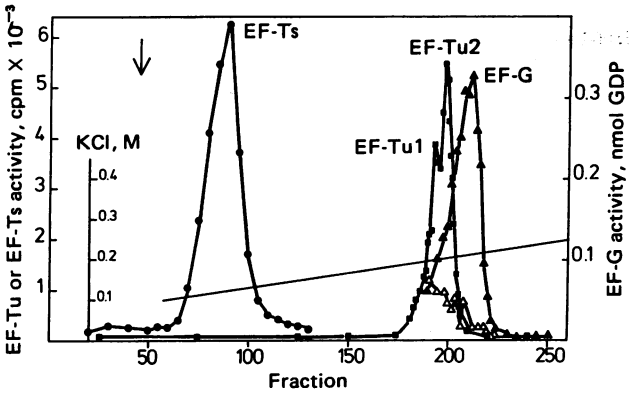

FIG. 1. Separation of the elongation factors of *E. coli* on a DEAE-Sephadex A50 column. The large preparative DEAE-Sephadex A50 column (3.4 × 50 cm) was loaded with about 2000 mg of protein. The column was first washed with low salt gradient buffer and then developed with 2.5 liters of linearly increasing salt gradient (0.1–0.4 M KCl) containing 20 mM Tris·HCl (pH 7.4 at 0°), 10 mM $Mg(OAc)_2$, 10 μM GDP, and 5 mM 2-mercaptoethanol. The flow rate was 40 ml/hr, and 250-drop fractions were collected. ●, EF-Ts activity; ■, EF-Tu activity; ▲, ribosome-dependent GTPase activity; △, ribosome-independent GTPase activity; ——, KCl concentration. The arrow shows the position where bulk GDP is released from the column.

at 0°), 160 mM NH_4Cl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.4 mg of poly(U) (Miles), 50 nmol of GTP, 50 μg of [^{14}C]Phe-tRNA (~300 mCi/mmol) (10), 2 μg of EF-Ts, and 3 μg of EF-G. The reaction was started by the simultaneous addition of [^{14}C]-Phe-tRNA and GTP. Units of EF-Tu and EF-Ts activities are all defined elsewhere (9).

Gel Systems. The sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide gel of Laemmli (13) was prepared as described but with the addition of 6 M urea to the stacking and separation gels as well as to the sample buffer (14). The urea/polyacrylamide gels at high pH (8.7) and low pH (4.5) were prepared as described (15, 16), but with the addition of 8 M urea to all buffers. The isoelectric focusing gel system used was prepared according to O'Farrell (17) for his first dimension. The polyacrylamide disc electrophoresis, our so-called native gel system, was performed as described (18). The molecular weights of EF-Tu1 and EF-Tu2 were determined by using the modified Laemmli gel system described above. Molecular weight standards were bovine serum albumin, aldolase, lactate dehydrogenase, and chymotrypsinogen (all from Serva) and EF-Ts (19, 20).

Protein Determination. All through the preparation, protein determinations were carried out as described by Ehresmann *et al.* (21).

RESULTS

We have found that the procedure of Arai *et al.* (9) for the separation of the *E. coli* elongation factors is reproducible with one exception. Fig. 1 shows a typical standard chromatographic profile. The peaks of EF-Ts, EF-Tu, and EF-G emerge in that order. The feature that differs from the pattern published by Arai *et al.* (9) is the splitting of the peak of EF-Tu activity into two peaks. We refer to the two components as EF-Tu1 and EF-Tu2, in order of elution. In order to establish the significance of this observation, we first carried out some controls to rule out trivial artifacts.

Because GDP is in the column buffer for the dissociation of EF-Tu and EF-Ts, and because GDP itself is an anion, we wished to be sure that the splitting was not due to the breakthrough of the GDP at or near the position of the EF-Tu peak. In a separate smaller scale experiment, the column was run with [^{3}H]GDP in the elution buffer. The position at which the GDP radioactivity appeared in the eluate is indicated by the arrow in Fig. 1. Thus, the splitting of the EF-Tu activity peak was not a consequence of the elution of GDP at the same point.

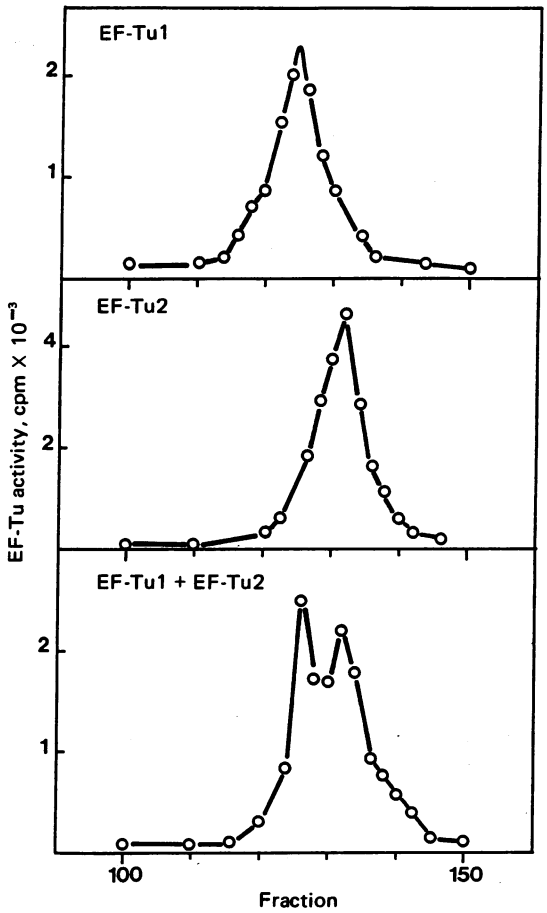

FIG. 2. Rechromatography of the two forms of EF-Tu. One-quarter of the EF-Tu1 and EF-Tu2 pools from a 1.2 × 20 cm DEAE-Sephadex A50 (fractions 130–155 and 167–185, respectively, from Fig. 3) were rechromatographed individually or as a mixture on a 0.6 × 10 cm DEAE-Sephadex A50 column, run as described in Fig. 1, but with the appropriate reduction of the buffer volumes. The flow rate was 1.5 ml/hr, and 0.25-ml fractions were collected.

Other possible kinds of chromatographic artifacts were ruled out by rechromatography. Again, a scaled-down version of the column shown in Fig. 1 was run, and the two peaks of EF-Tu activity were pooled separately. Aliquots of these pools were run again on analytical-scale DEAE-Sephadex A50 columns. The results are shown in Fig. 2. The EF-Tu1 and EF-Tu2 appeared in positions corresponding to the elution positions in the original profile. However, because the two positions peak were close, as an additional control to confirm their nonidentity, we ran a mixture of EF-Tu1 and EF-Tu2. Two peaks were seen again, at the expected positions. We therefore conclude that the peaks of EF-Tu activity correspond to two unique and distinguishable molecular species.

We also need to rule out artifactual splitting due to the binding of some other ligand. Fig. 3A (open symbols) shows that there is no EF-Ts activity in either EF-Tu peak. We are therefore not dealing with an incompletely dissociated EF-Tu–EF-Ts complex. Furthermore, $NaDodSO_4$ gels of the column fractions showed no protein band at a position corre-

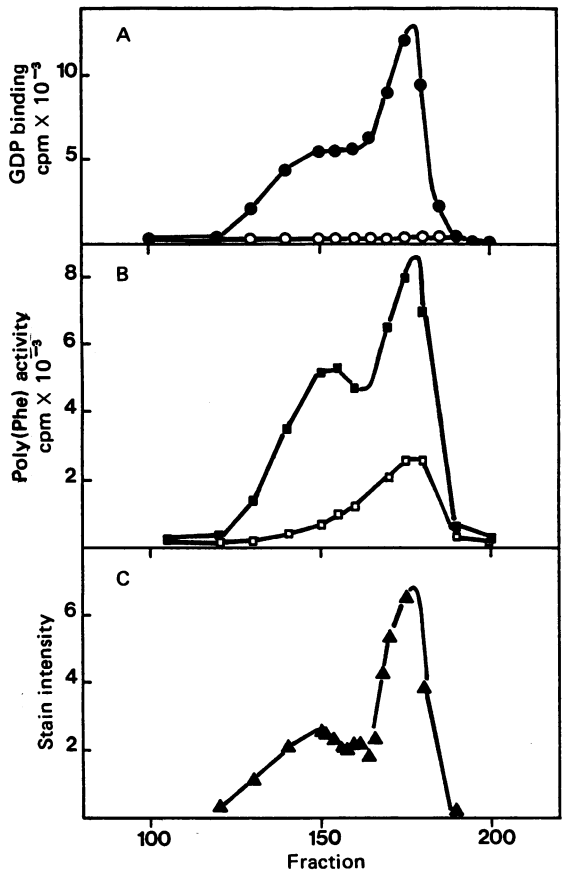

FIG. 3. EF-Tu profile as measured with different assays. About 110 mg of protein was run on a DEAE-Sephadex A50 column (1.2 × 20 cm) as described in Fig. 1 with the appropriate reduction of the buffer volumes. The flow rate was 6 ml/hr and 1-ml fractions were collected. (*A*) Fractions (5 μl) were assayed for EF-Tu activity in the absence (○) or presence (●) of 1.2 μg of EF-Ts by the GDP-binding assay described in *Materials and Methods* except that the incubation was only for 30 sec. (*B*) Fractions (5 μl) from the same column were assayed for EF-Tu activity as poly(Phe) synthesis activity in the absence (□) or in presence (■) of 3 μg of EF-G. (*C*) An aliquot of each fraction (5 μl) was run on a urea/$NaDodSO_4$/polyacrylamide gel (see Fig. 4). The gels were scanned and the area under the EF-Tu band on the scan was cut off and weighed (the weight of the paper was proportional to the EF-Tu stain intensity as shown by a control experiment).

sponding to the known molecular weight of EF-Ts (20). This can be seen in the gels of the individual DEAE-Sephadex fractions shown in Fig. 4. The presence of two peaks is therefore not the result of an EF-Tu–inactive EF-Ts complex. In addition, the splitting cannot be simply accounted for by a complex with EF-G whose existence was also once suspected (22, 23). The EF-G present in the EF-Tu2 peak is entirely consistent with the leading edge of the EF-G peak, in terms of GTPase activity (Fig. 1), of poly(Phe) activity determined in the absence of added EF-G (Fig. 3*B*, open symbols) and of distribution of the EF-G protein as can also be seen in the gel of Fig. 4.

Table 1. Stoichiometry of GDP binding

	% purity	GDP/Tu, mol/mol
EF-Tu 1	24	0.83
EF-Tu 2	60	0.72

The EF-Tu fractions after the Sephadex G-100 step of purification were run on a urea/$NaDodSO_4$/polyacrylamide gel (Fig. 5). The purity and the amount of EF-Tu protein were calculated from integration of the densitometry of the gels. The GDP binding activity was measured in the presence of excess Ts.

The evidence presented so far shows that there are two peaks of EF-Tu as measured by GDP binding activity. Fig. 3*B* (closed symbols) shows that both peaks have poly(Phe) activity in the presence of excess EF-G and EF-Ts. Fig. 3*C* shows the distribution of the EF-Tu protein based from stain intensity of the $NaDodSO_4$ gels of Fig. 4. This was done by carrying out densitometry of the gel and integrating the stain intensity of the peak identified as EF-Tu. Thus, it can be seen that there are two peaks of EF-Tu, by all criteria.

In addition to having the same profiles, EF-Tu1 and EF-Tu2 also both bound GDP stoichiometrically (Table 1). This was done by measuring the GDP binding activity of each fraction and the amount of EF-Tu protein in the fraction calculated from scans of $NaDodSO_4$ gels (Fig. 5).

The two forms also cannot be distinguished on the basis of molecular weight. This was apparent from $NaDodSO_4$ gels (Fig. 4), from the Sephadex G-100 column elution, and, in more detail, from $NaDodSO_4$ gels of the G-100 fractions (Fig. 5). From this, the molecular weight was calculated to be 45,500 ± 1000, consistent with published data (9, 24, 25). Jacobson and Rosenbusch (26) found that they could split off a 7000 dalton fragment without affecting the activity of EF-Tu, although the data apparently are controversial (24). We found the same pattern of tryptic fragments for both EF-Tu1 and EF-Tu2 (data not shown). The difference between EF-Tu1 and EF-Tu2 was therefore not due to such a proteolysis product.

It was not possible to distinguish between the two forms of EF-Tu by any of the following additional criteria: analytical

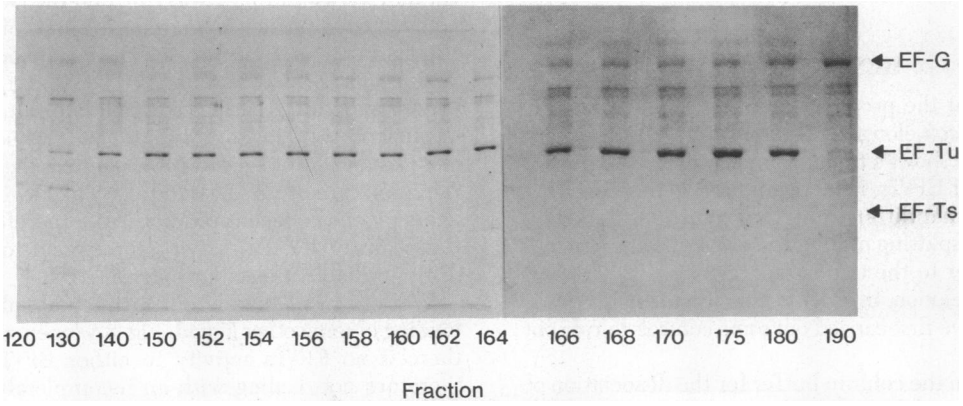

FIG. 4. $NaDodSO_4$ gel electrophoresis of fractions from DEAE-Sephadex A50 column. An aliquot (5 μl) of each fraction from the column described in Fig. 3 was run on a urea/$NaDodSO_4$/polyacrylamide gel.

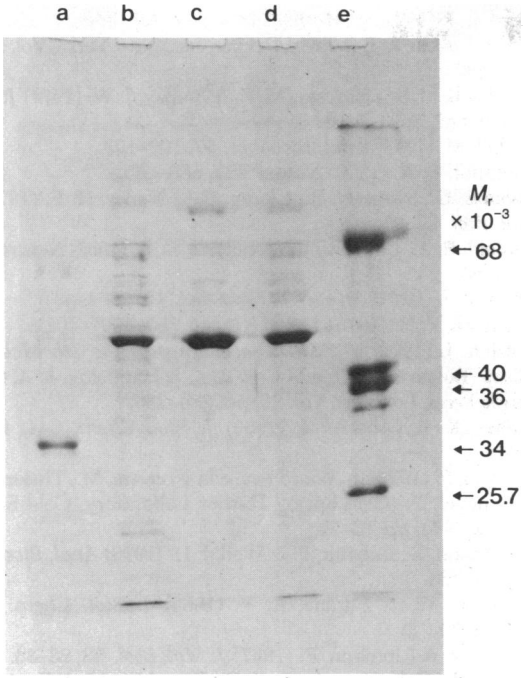

FIG. 5. Molecular weight of both forms of EF-Tu. An aliquot of EF-Ts (1 μg) (a), EF-Tu1 and EF-Tu2 after the Sephadex G-100 filtration step (2 μg) (b and c, respectively), and a mixture of both (d) was run on urea/NaDodSO$_4$/polyacrylamide gel together with standards (e). The molecular weights (M_r) of the standards and of EF-Ts are shown at the right.

electrofocusing, urea/polyacrylamide gel electrophoresis at high or low pH, and native gels with or without Mg^{2+} and GDP (data not shown).

If EF-Tu1 and EF-Tu2 have any functional significance, it should be possible to distinguish between them by some operational criterion other than column chromatography. We therefore investigated whether one or the other form was preferentially bound to ribosomes (Fig. 6). The ribosomes were pelleted harder, as described in the legend to Fig. 6, in order to get a clear partition between ribosomal and soluble material. Under these conditions, the EF-Tu1 peak apparently disappears whereas EF-Tu2 remains in the supernatant. The centrifugation time of the S100 fraction has a drastic effect on the ratio EF-Tu1/EF-Tu2 (Fig. 6 *inset*). However, addition of washed ribosomes to a preparation of EF-Tu2 did not generate two peaks of activity (data not shown). The observed heterogeneity is therefore not due to the presence of ribosomes in the preparation *per se*. The finding of a selective depletion of EF-Tu1 from the supernatant fraction with longer times of centrifugation implies, but does not prove, a selective binding to ribosomes. It does, however, provide an additional criterion that EF-Tu1 and EF-Tu2 are different.

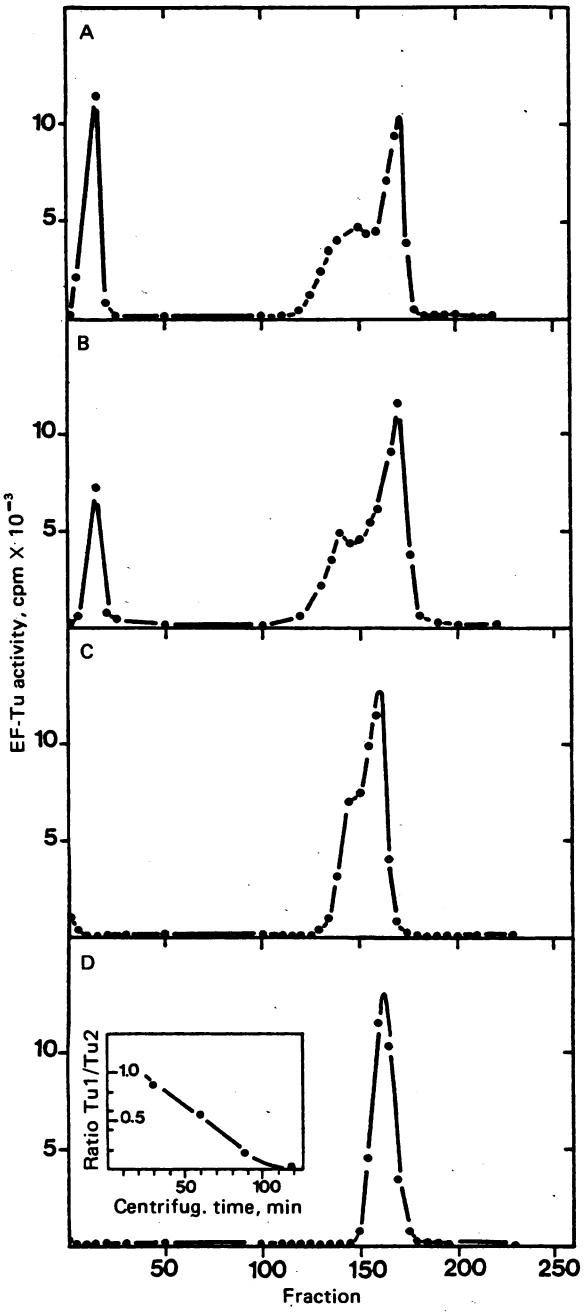

FIG. 6. Affinity of EF-Tu1 for the ribosomal pellet. The S100 fraction described in *Materials and Methods* was centrifuged for 30, 60, 90, or 120 min (*A*, *B*, *C*, or *D*, respectively) at 50,000 rpm in a Beckman Ti50 rotor. From the ammonium sulfate precipitates of the different S100 fractions, a volume corresponding to the same amount of wet cells was loaded on a 1.2 × 20 cm DEAE-Sephadex A50 column (about 100 mg of protein) run as described in Fig. 3. EF-Tu was assayed as GDP-binding activity. (*Inset*) Ratio Tu1/Tu2 was calculated from the total activity under the Ef-Tu1 and EF-Tu2 peaks, respectively, and plotted as a function of the centrifugation time.

DISCUSSION

The finding that EF-Tu1 is selectively depleted from the S100 fraction at longer centrifugation times suggests that it is selectively bound to the ribosomal fraction. This could be explained by two different kinds of model: (*i*) some modification of the EF-Tu, in addition to the hydrolysis of GTP, must take place before the EF-Tu can leave the ribosome during the normal course of the polypeptide chain elongation cycle; or (*ii*) there exist two subpopulations of EF-Tu that have inherently different affinities for the ribosome. This may be interesting in the light of the finding by Grasmuk *et al.* (27) that the analogous eukaryotic factor from Krebs II ascites cells, EF1, binds to ribosomes independently of the presence of nucleotide and aminoacyl-tRNA. The results obtained in ribosome binding experiments in *E. coli* may depend on whether the preparation contains predominantly EF-Tu1 or EF-Tu2. A further parallel with eukaryotic systems may be the almost universal finding in eukaryotes of heavy and light forms of EF1 (28, 29). Such

heterogeneity has never been observed in prokaryotes; the heterogeneity described here may have some analogy.

The parallelism between our observations and the finding of two distinct structural gene loci for EF-Tu (4) is compelling. Furano (30) has emphasized the conservation of the two gene products, but a slight difference of one spot in the tryptic fingerprint has not escaped his attention (30). Such a difference may be sufficiently subtle to escape detection by most analytical procedures but may still result in the chromatographic behavior reported here. This remains to be seen.

The question of the nature of the difference between the two forms of EF-Tu is unresolved at this point. We need to explain the apparent paradox that the forms are separable on the DEAE-Sephadex column yet are indistinguishable by all of the other analytical procedures used. Our working hypothesis is that the two forms have a small difference in primary structure—perhaps a neutral amino acid replacement—which results in an alternative conformation.

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