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# A Functionally Active Tryptic Fragment of *Escherichia coli* Elongation Factor Tu†

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ABSTRACT: Mild tryptic hydrolysis of native elongation factor Tu from *Escherichia coli* yields a unique fragment which lacks some 65 residues at the amino-terminal end of the protein. This fragment retains the capacity to bind GDP, to form a ternary complex with GTP and aminoacyl-tRNA and to stimulate in vitro protein synthesis. Like the native protein, the fragment renatures from a random coil conformation to a fully functional form. These results show that the fragment is an independent structural and functional unit.

In a study of the apparent membrane association of a major protein from *Escherichia coli* (Jacobson et al., 1976), we noted that mild tryptic hydrolysis nearly quantitatively converted this polypeptide (molecular weight 44 000) to a species with an apparent mass of 37 000 daltons. More recently, we have identified this protein as elongation factor Tu (Jacobson and Rosenbusch, 1976). In its role in protein synthesis, EF-Tu[1] interacts with a number of other proteins and substrates (for

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[1] Abbreviations used are: EF-Tu, EF-Ts, and EF-G, protein synthesis elongation factors Tu, Ts, and G, respectively; EF-Tu$^t$, the 37 000-dalton tryptic fragment of EF-Tu; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

a review, cf. Lucas-Lenard and Beres, 1974). It also constitutes a subunit of an RNA phage replicase (Blumenthal et al., 1972), it may play a role in the regulation of transcription (Travers et al., 1970; Travers, 1973), and it is apparently partially associated with the membrane. In view of this multifunctionality, and of the concept that different functions may be carried out by autonomous regions in a protein (Kirschner and Bisswanger, 1976), it was of interest to determine the functional properties of this fragment. In this report, we show that the 37 000-dalton fragment of EF-Tu arises from the cleavage of a 7000-dalton segment from the amino-terminal end of the native protein. The large fragment is an apparently homogeneous polypeptide species and retains all of the functions which native EF-Tu performs in protein synthesis. These results raise the question of what functions the amino-terminal segment may possess.

## Experimental Procedures

*Preparation of Elongation Factors, Ribosomes, and tRNA.* Crystalline EF-Tu–GDP complex was prepared from *E. coli* BE according to Miller and Weissbach (1970) or Arai et al. (1972). Assays during purification were performed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. To obtain a preparation containing EF-G and EF-Ts activities (free of EF-Tu), cells were suspended in an equal weight of 50 mM Tris-HCl, pH 7.8, containing 10 mM $MgCl_2$ and 1 mM dithiothreitol (buffer A), passed through a Manton-Gaulin mill at 7000 psi, and centrifuged sequentially at 30 000*g* for 30 min and at 100 000*g* for 3 h. The supernatant was heated at 55 °C for 15 min to destroy EF-Tu activity (Rohrbach et al., 1974) and clarified by centrifugation. The resulting solution was used in the in vitro phenylalanine polymerization assay for EF-Tu (see below).

High-salt-washed ribosomes and [$^{14}$C]phenylalanyl-tRNA were prepared from *E. coli* BE according to Nishizuka et al. (1968). Unlabeled phenylalanyl-tRNA was prepared by the same method and the charging efficiency estimated from that obtained under the same conditions with [$^{14}$C]phenylalanine.

*Preparation and Purification of EF-Tu$^t$.* Solutions of EF-Tu–GDP complex (1–2 mg per ml) in buffer A were incubated at 25 °C with freshly dissolved *N*-tosyl-L-phenylalanine chloromethyl ketone treated trypsin (products of Serva or Worthington) at a protease concentration of 2% (by weight) of that of EF-Tu. The exact reaction time for optimal yields of EF-Tu$^t$ varied between 2 and 5 min, depending on the specific activity of the trypsin used. It was determined for each preparation by measuring the time course of proteolysis of EF-Tu as shown in Figure 1. The reaction was stopped by addition of a threefold molar excess of bovine pancreatic trypsin inhibitor (a kind gift of Dr. Jürgen Engel) over the concentration of trypsin. EF-Tu$^t$ was purified from this reaction mixture by Sephadex G-150 chromatography in buffer A at 4 °C.

*Assays.* The binding of GDP to EF-Tu was assessed by the nitrocellulose filter technique of Miller and Weissbach (1970) or by a modified procedure (Suter and Rosenbusch, 1976). Binding mixtures contained 10 pmol of elongation factor and 20–400 nM [$^{14}$C]GDP in 1 ml of buffer A. Specific activities were corrected for unlabeled protein-bound GDP. The mixtures were incubated 15 min at 25 °C before filtration. Control incubations for 30 min yielded the same results. The data were analyzed by the method of Scatchard (1949) after correction of the amount of ligand bound to the filters for appropriate blank values (Suter and Rosenbusch, 1976).

In vitro polymerization of phenylalanine was determined under conditions described by Lucas-Lenard and Lipmann (1966). Reaction mixtures contained 20 μg of poly(uridylic acid), 80 μg of [$^{14}$C]phenylalanyl-tRNA, 200 μg of high-salt-washed ribosomes, 20 μl of a preparation containing EF-G and EF-Ts activities (see above), and 20 μg of EF-Tu or EF-Tu$^t$ in a total volume of 500 μl of buffer (50 mM Tris-HCl, pH 7.4, 0.16 M $NH_4Cl$, 10 mM $MgCl_2$, and 1 mM dithiothreitol). The mixture was preincubated for 10 min at 30 °C, and the reaction was started by addition of GTP to a final concentration of 0.2 mM. Aliquots (100 μl) were taken at appropriate times, pipetted into 1 ml of 5% trichloroacetic acid, and heated at 90 °C for 15 min. The solutions were cooled, filtered on Whatman GF/C glass fiber filters, and washed two times each with 1 ml of 5% trichloroacetic acid and 1 ml of 95% ethanol. The filters were air-dried and counted in scintillation vials containing 10 ml of scintillation fluid (Takacs and Rosenbusch, 1975). Under these conditions, the assay always gave a linear relationship between polyphenylalanine synthesized and the concentration of EF-Tu.

Formation of the ternary complex between EF-Tu or EF-Tu$^t$, GTP, and aminoacyl-tRNA was assayed according to the procedure described by Miller and Weissbach (1974). Phosphoenolpyruvate (Boehringer) and pyruvate kinase (Fluka) were added to the assay mixtures as described to convert all GDP to GTP.

*Denaturation–Renaturation Experiments.* EF-Tu and EF-Tu$^t$ were renatured from 8 M urea solutions by two methods. The dilution procedure of Blumenthal and Landers (1976) was used when GDP binding was determined. The proteins (0.5 mg per ml in buffer A) were denatured by addition of solid urea to a final concentration of 8 M. After incubation for 60 min at 25 °C, the solutions were diluted with 10 volumes of buffer B (50 mM Tris-HCl, pH 8.0, containing 10 mM $MgCl_2$, 1 mM EDTA, 0.15 M $(NH_4)_2SO_4$, 20% glycerol, and 70 mM 2-mercaptoethanol), kept at 25 °C for 4 h, and then tested for GDP binding as described above. The second procedure was used when EF-Tu and EF-Tu$^t$ were assayed for stimulation of in vitro polyphenylalanine synthesis. Denaturation was accomplished by dialysis for 4 h against buffer B containing 8 M urea. Samples (100 μl) of EF-Tu$^t$ were then subjected to gel filtration on Sephadex G-150 (1 × 20 cm) in buffer B containing 8 M urea to remove any residual, noncovalently bound peptides. Peak fractions were pooled and bovine pancreatic trypsin inhibitor (20 μg per ml) was added. To this solution, and to control samples which were denatured but not chromatographed, 1 mM GDP was added and the samples were then dialyzed overnight at 20 °C against buffer B. Final dialysis at 4 °C for 5 h was against twofold concentrated phenylalanine polymerization buffer (see above).

*Other Procedures.* Conditions for sodium dodecyl sulfate electrophoresis in polyacrylamide slab gels have been described previously (Takacs and Rosenbusch, 1975). The procedures for amino acid analyses and for the recording of circular dichroism spectra were those quoted in detail previously (Rosenbusch, 1974; Griffin et al., 1972). Protein concentrations were determined according to Lowry et al. (1951). Digestions of EF-Tu and EF-Tu$^t$ with carboxypeptidases B (Ambler, 1967) and P (Isobe et al., 1976) were kindly performed by Drs. T. Isobe and A. Tsugita. Cleavage of EF-Tu and EF-Tu$^t$ at cysteine residues was performed as described by Jacobson et al. (1973) using the two-step modification procedure with 5,5′-dithiobis(2-nitrobenzoic acid) and undiluted K$^{14}$CN, with the exception that the reactions were carried out in 0.5% sodium dodecyl sulfate as denaturant. Cleavage products were

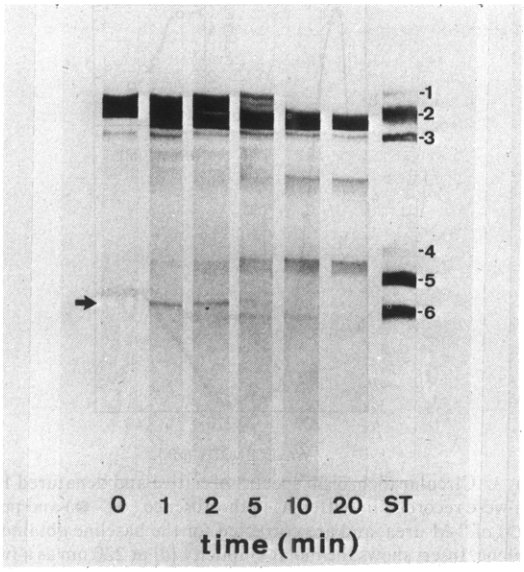


FIGURE 1: Kinetics of hydrolysis of EF-Tu by trypsin. Native EF-Tu was treated with trypsin under conditions described in Experimental Procedures. Aliquots were taken at the indicated times, pipetted directly into boiling dodecyl sulfate sample buffer, and analyzed on a polyacrylamide gradient gel (10–25% polyacrylamide) in the detergent. The arrow marks the position of the 7000-dalton peptide visible at 1 and 2 min. Molecular weight standards at the right are: (1) ovalbumin (45 000); (2) aldolase (40 000); (3 and 4) aspartate transcarbamoylase catalytic chain (33 500) and regulatory chain (17 000), respectively; (5) lysozyme (13 500); (6) bovine pancreatic trypsin inhibitor (6500). The EF-Tu preparation used contained a slight contamination, probably EF-Ts, which migrated just ahead of EF-Tu$^t$ on the gel.

analyzed directly by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. They were quantitated by stain elution with dimethyl sulfoxide from bands cut from the gel. The incorporated label was measured from duplicate samples dissolved with $H_2O_2$ as described previously (Suter and Rosenbusch, 1975).

*Other Materials.* $^{14}$C-labeled GDP, GTP, phenylalanine, and KCN were products of Amersham (England), and unlabeled nucleotides were products of Merck. Poly(uridylic acid) and urea were purchased from Serva and guanidinium chloride (Ultrapure) from Schwarz/Mann. All other chemicals used were analytical grade.

## Results

*Kinetics of Hydrolysis of EF-Tu by Trypsin.* Figure 1 shows the time course of the tryptic hydrolysis of native EF-Tu–GDP complex (molecular weight 44 000), analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Rapid conversion of EF-Tu to a relatively trypsin-resistant intermediate, which we have designated EF-Tu$^t$ (molecular weight 37 000), is nearly complete after 5 min under the conditions of the experiment shown. At earlier times (1–2 min after the onset of the reaction), a peptide with an apparent mass of 7000 ± 1000 daltons, indicated by the arrow in Figure 1, can be seen transiently. The kinetics of appearance of this peptide, as well as its size, suggest that the primary event in the conversion of EF-Tu to EF-Tu$^t$ is the cleavage of a trypsin-sensitive bond which is approximately 65 amino acid residues removed from one end of the polypeptide chain.

It is also apparent from Figure 1 that times of hydrolysis longer than 5 min lead to the rapid degradation of the 7000-dalton segment. Furthermore, two new polypeptide bands appear between 5 and 20 min of hydrolysis. Their sizes (24 000 and 14 000 daltons) suggest that they are generated from

TABLE I: Analysis of Cysteine Cleavage Products of EF-Tu and EF-Tu$^t$.

| Protein[a] | Mol Wt | Cyanide Incorp per Polypeptide Chain (mol/mol) |
|---|---|---|
| EF-Tu | | |
| Uncleaved[b] | 44 000 | 0.07[c] |
| Product 1 | 29 500 | 0.07 |
| Product 2 | 14 000 | 0.70 |
| EF-Tu$^t$ | | |
| Uncleaved[b] | 37 000 | 0.08 |
| Product 1 | 23 500 | 0.06 |
| Product 2 | 14 000 | 0.71 |

[a] Quantitation of cleavage products and incorporated radioactivity was performed from dodecyl sulfate gel electropherograms, as described in Experimental Procedures. [b] Cleavage at cysteinyl residues was incomplete and occurred primarily at one specific site. Possible reasons for this incomplete cleavage are manifold (cf. Degani and Patchornik, 1974), but they are not relevant in the present context. [c] The small amount of label in uncleaved material and in product 1 is probably due to a reactive contaminant (possibly [$^{14}$C]cyanate).

cleavage of EF-Tu$^t$ at a single or closely adjacent sites in the molecule. Due to the different rates of the primary and subsequent tryptic cleavage events, a careful control of the reaction time allowed us to obtain EF-Tu$^t$ in essentially pure form.

*Structural Autonomy and Location of the EF-Tu$^t$ Fragment.* When the reaction products of the tryptic degradation of EF-Tu are chromatographed on Sephadex G-150 under nondenaturing conditions, the elution position of EF-Tu$^t$ is similar to that of EF-Tu (data not shown). This result suggests that this large fragment retains a compact, globular structure. Gel electrophoretic analyses in sodium dodecyl sulfate confirm quantitative conversion and indicate that substantial amounts of oligopeptide fragments are not bound to EF-Tu$^t$ after purification by gel filtration. Stepwise chemical degradation (Weiner et al., 1972) of its amino-terminal end yielded the sequence:

$$H_2N\text{-Gly-Ile-Thr-Ile-}\ldots$$

This result shows that the fragment has a new, unique amino-terminus, since that of the parent EF-Tu is blocked (Blumenthal et al., 1972; Wade et al., 1975; Jacobson and Rosenbusch, 1976).

To localize the tryptic cleavage site(s) in EF-Tu, we did the following experiments. Digestion of EF-Tu$^t$ with carboxypeptidases B or P (see Experimental Procedures) did not yield significant amounts of either lysine or arginine. This result suggests that the carboxy-terminal end of EF-Tu$^t$ is the same as that of the native protein. To confirm that the large fragment arises through the loss of an amino-terminal peptide from the parent protein, we subjected EF-Tu and EF-Tu$^t$ to a chemical cleavage method which splits polypeptides specifically at cysteinyl residues (Jacobson et al., 1973). With this procedure, all peptides except for that derived from the original amino-terminus are labeled if [$^{14}$C]cyanide is used in the reaction. The gel electrophoretic analysis, summarized in Table I, indicates that cleavage of EF-Tu and EF-Tu$^t$ was partial and occurred predominantly at a unique cysteinyl residue, thus yielding two major fragments for either protein. The sum of the respective masses of the arising peptides accounts for those of the parent proteins. The larger fragments from each protein (product 1) are not significantly labeled and are, therefore,

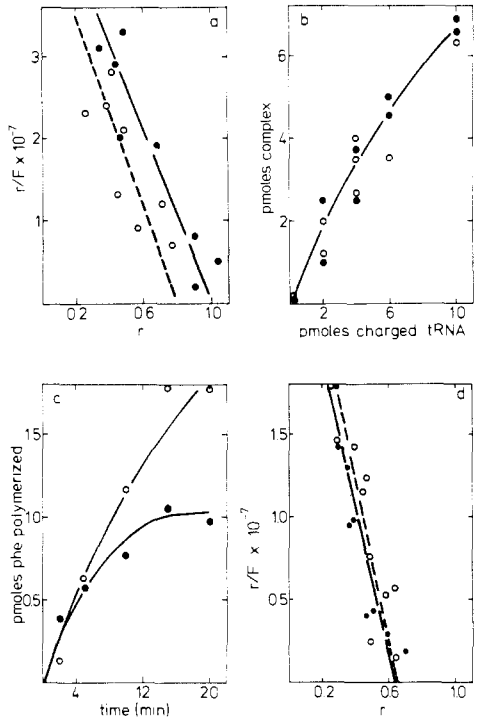


FIGURE 2: A comparison of the functional properties of EF-Tu and EF-Tu$^t$. (a) Scatchard plot (Scatchard, 1949) of GDP binding to EF-Tu (●) and EF-Tu$^t$ (○). The lines shown are derived from least-squares analyses of the data sets. (b) Formation of the ternary complex between elongation factor, GTP, and aminoacyl-tRNA as a function of phenylalanyl-tRNA concentration. (c) Stimulation of polyphenylalanine synthesis by EF-Tu and EF-Tu$^t$. Units on the ordinate are for 100 μl of reaction mixture as described in Experimental Procedures. (d) Scatchard plot of GDP binding to EF-Tu and EF-Tu$^t$ renatured by the dilution method (see Experimental Procedures). The data were analyzed as in a.

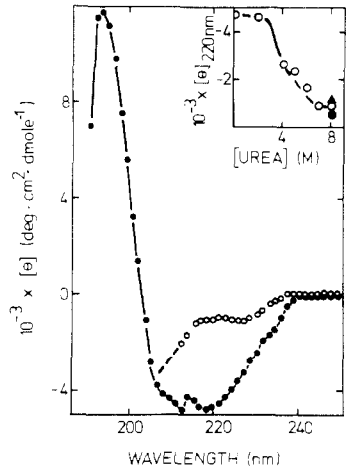


FIGURE 3: Circular dichroism spectra of native and denatured EF-Tu. Spectra were recorded in buffer A, in the absence (●—●) and presence (○- - -○) of 8 M urea, and are corrected for the baseline obtained with buffer alone. Insert shows the molar ellipticity [θ] at 220 nm as a function of the urea concentration for EF-Tu (○). ▲ indicates the value for EF-Tu$^t$ in 8 M urea and ● the value for EF-Tu in 8 M guanidinium chloride.

derived from the amino-terminal ends of the two polypeptides. They differ in their masses by 6000 daltons, a result which is in reasonable agreement with the difference in size between EF-Tu and EF-Tu$^t$. The smaller fragments from each protein (product 2) are labeled and are apparently identical in size. These results confirm that the primary tryptic cleavage site is approximately 65 residues from the amino-terminus of EF-Tu and are consistent with the observation that no new carboxy-terminal end could be detected in EF-Tu$^t$.

*Functional Properties of EF-Tu$^t$.* The results of studies investigating the protein biosynthetic functions of EF-Tu$^t$ are shown in Figure 2. Trypsin-modified molecules bind GDP with an affinity (20 nM) and a stoichiometry (0.8 mol per mol of protein) similar to that of unmodified EF-Tu (Figure 2a). In addition, the ability of the fragment to form a ternary complex with GTP and aminoacyl-tRNA is unimpaired (Figure 2b). Finally, EF-Tu$^t$ is fully competent with respect to the in vitro polymerization of phenylalanine in the presence of ribosomes, [$^{14}$C]phenylalanyl-tRNA, GTP, and the other two elongation factors (Figure 2c).

*EF-Tu$^t$ is an Autonomous Unit of Folding.* To investigate whether EF-Tu$^t$ contains the information necessary for its proper folding, the fragment was denatured in 8 M urea. Subsequently, the chaotropic agent was removed by a procedure allowing successful renaturation of native EF-Tu (Blumenthal and Landers, 1976). Since we wished to establish that the conditions used led to complete denaturation both of the native protein and of the fragment, it was necessary first to examine the extent of unfolding of the two polypeptides. The circular dichroism spectra of EF-Tu in the presence and absence of 8 M urea are shown in Figure 3. The spectral changes observed upon addition of the chaotropic agent and the titration curve of the molar ellipticity at 220 nm as a function of the urea concentration suggest that the denaturation of EF-Tu is complete under these conditions. Although some residual absorption between 220 and 235 nm may be noted in the presence of 8 M urea, the titration curve shows that 8 M guanidinium ions do not change the ellipticity further. It is, therefore, reasonable to assume (Tanford, 1968) that both EF-Tu and EF-Tu$^t$ exist in random coil conformations in 8 M urea.

Removal of the chaotropic agent results in the renaturation of EF-Tu$^t$. Figure 2d shows that reactivation occurs to the same extent (approximately two-thirds of the initial activity) as EF-Tu, as measured by their respective abilities to bind GDP. The affinities for GDP of renatured EF-Tu and EF-Tu$^t$ are identical (20 nM) with those of the undenatured proteins (Figure 2a). This observation allowed us to further confirm experimentally that oligopeptides derived from the amino-terminal segment degraded by trypsin are not required for either the refolding of EF-Tu$^t$ or for its activity. To this end, we chromatographed EF-Tu$^t$ on Sephadex G-150 in 8 M urea before renaturation. Gel electrophoresis in sodium dodecyl sulfate demonstrated that all traces of small peptides had been removed by the gel filtration step. Table II shows that the specific activity of this preparation in the phenylalanine polymerization assay is similar to that of unchromatographed, renatured EF-Tu$^t$. We therefore conclude that EF-Tu$^t$ is also an independent unit of folding and, thus, can be considered autonomous in a structural as well as in a functional sense.

## Discussion

Tryptic hydrolysis of native EF-Tu to form EF-Tu$^t$ occurs via a rapid cleavage reaction at a site approximately 65 residues from the amino-terminal end of the protein. This was established from the appearance, at early times of proteolysis, of a peptide with a mass of 7000 daltons which we have shown to be derived from the original amino terminus of EF-Tu. Because of its very high sensitivity to trypsin, this cleavage site is likely exposed to the solvent in EF-Tu. Since further hydrolysis of the resulting EF-Tu$^t$ is slow, trypsin-sensitive bonds in this fragment must be relatively unavailable to the enzyme. Hydrolysis into two smaller fragments does, however, occur (cf.

TABLE II: Relative Activities of Native and Renatured EF-Tu and EF-Tu$^t$ in Polyphenylalanine Synthesis.

| Protein | Rel Sp Act.[a] |
|---|---|
| Native EF-Tu | 1.0 |
| Undenatured EF-Tu$^t$ | 0.84 |
| EF-Tu renatured from 8 M urea | 0.93 |
| EF-Tu$^t$ renatured from 8 M urea | 0.85 |
| EF-Tu$^t$ renatured after Sephadex G-150 chromatography in 8 M urea[b] | 0.90 |

[a] The activity of native EF-Tu is set to a value of 1.0, and other activities are normalized accordingly. Actual specific activities in the phenylalanine polymerization assay have varied in our hands. For EF-Tu, it was between 0.2 and 0.8 nmol of phenylalanine polymerized per mg of protein in 5 min, depending on the ribosome and phenylalanyl-tRNA preparations used. [b] For the conditions of chromatography, cf. Experimental Procedures.

also Nakamura et al., 1975). Our preliminary data indicate that this cleavage reaction, which eventually goes to completion, inactivates the protein with respect to its ability to function in the polymerization of phenylalanine. End-group analyses of EF-Tu$^t$ show that it has a unique amino-terminal sequence and probably retains the original carboxy-terminal end. The amino-terminal peptide (7000 daltons) is rapidly degraded and is, therefore, likely to be freely available to the protease after its cleavage from the native molecule.

Conversion of EF-Tu to EF-Tu$^t$ does not cause substantial changes in the conformation of the large fragment since it does not affect its ability to function as an elongation factor. Furthermore, direct measurements of the affinity of EF-Tu$^t$ for GDP and of its ability to form a ternary complex with GTP and aminoacyl-tRNA do not show significant differences when compared with the native protein. Since the fragment functions at least as well in phenylalanine polymerization, EF-Tu$^t$ also appears to retain sites of recognition for EF-Ts and for the proteins to which it binds on the ribosome (L7/L12; cf. Möller, 1974). The missing polypeptide segment is not required for EF-Tu$^t$ to refold from a random coil state to an active conformation. We confirmed that no small peptides produced by tryptic hydrolysis are necessary either for the activity or the refolding of EF-Tu$^t$ by subjecting the tryptic reaction mixture to gel filtration in 8 M urea before renaturation. EF-Tu$^t$ may, therefore, be considered a structurally and functionally autonomous unit which also contains the information necessary for its proper folding. Its properties thus conform to the definition of an autonomous structural region or to that of a "domain" (Wetlaufer, 1973; Kirschner and Bisswanger, 1976). Although it remains to be determined whether EF-Tu$^t$ may not contain more than one functional unit, the EF-Tu molecule can be pictured as consisting of at least two topographically distinct regions, a small amino-terminal and a large carboxy-terminal segment which are connected by a trypsin-sensitive stretch of the polypeptide chain.

Our observations raise the question of what role the amino-terminal region plays in the EF-Tu molecule. Since EF-Tu is a multifunctional protein (Blumenthal et al., 1972; Travers et al., 1970; Travers, 1973), it will be interesting to study the properties of EF-Tu$^t$ with respect to these activities. In addition, we have recently shown that a fraction of EF-Tu in *E. coli* may be associated with the plasma membrane in vivo (Jacobson et al., 1976), an observation which may suggest another, as yet unknown, function for the protein. A comparison of a preliminary amino acid composition of EF-Tu$^t$ with that of the undegraded factor indicates that their hydrophobicity coefficients (Heller, 1968) are similar. It therefore appears unlikely that the amino-terminal fragment functions as anchoring domain of this protein in the membrane.

Several proteins have recently been reported to be hydrolyzed to at least partially functional fragments by mild proteolysis (Platt et al., 1973; Epely et al., 1976; for a review, cf. Kirschner and Bisswanger, 1976). Of particular significance in the present context is the observation of Skar et al. (1975) that native EF-G (molecular weight 74 000) from *E. coli* can also be rapidly cleaved by mild trypsin treatment to a species with a mass of 71 000 daltons which also retains the functional properties of the parent molecule. Since *E. coli* has been shown to have a protease with trypsin-like specificity (Pacaud and Uriel, 1971; Pacaud and Richaud, 1975), it is possible that the observed in vitro cleavage of either EF-Tu or EF-G could also take place in vivo. If such processing (Hershko and Fry, 1975) occurs, it would be of interest to determine whether this phenomenon, which in yeast has been shown to have regulatory significance (Holzer et al., 1973), has a similar function also in bacterial cells.

Acknowledgment

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# A Simple, Quantitative Approach to the Coupling of Photophosphorylation to Electron Flow in Terms of Proton Fluxes†

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ABSTRACT: A simple relationship between observed phosphorylation efficiencies (*P/e* ratios) and internal proton concentration in spinach chloroplast thylakoids has been derived. *P/e* ratios, varied by either changing the light intensity or by adding the energy transfer inhibitor, 4′-deoxyphlorizin, were found to change with internal proton concentration in accordance with this relationship. A quantitative prediction of the effect of uncouplers on the *P/e* ratio can probably also be made. By extrapolation of plots of observed *P/e* ratios against internal proton concentration divided by the overall rate of electron flow, a maximum intrinsic *P/e* of about 0.66 is obtained. Assuming that two protons appear inside thylakoids per electron transferred, a *P/e* ratio of 0.66 suggests that three internal protons are consumed for each ATP formed. Internal protons may be considered to be substrates for the phosphorylation reaction. Hill plots of phosphorylation rate vs. internal proton concentration also indicate that three protons are consumed for each ATP synthesized. Thus, the $H^+$ concentration gradient behaves quantitatively, as well as qualitatively, as if it is the connecting link between electron flow and phosphorylation in illuminated thylakoids.

Strong evidence supporting at least the basic postulates of the chemiosmotic interpretation (Mitchell, 1966) of the coupling of phosphorylation to light-driven electron flow in chloroplast thylakoids has been presented. Illuminated thylakoids catalyze a light-dependent, uncoupler-sensitive $H^+$ uptake (Neumann and Jagendorf, 1964), resulting in a pH differential of 3 units or more across thylakoids membranes (Rottenberg et al., 1972; Schuldiner et al., 1972; Portis and McCarty, 1973, 1974). Since pH differentials formed artificially across thylakoid membranes in the dark can serve as the driving force for ATP synthesis (Jagendorf and Uribe, 1966), it is logical to conclude that the pH differential generated by light-induced electron flow serves this purpose also.

Qualitatively, the pH differential ($\Delta$pH) was found to have properties consistent with its being the connecting link between electron flow and ATP synthesis. For example, phosphorylation and uncouplers (Portis and McCarty, 1974; Pick and Avron, 1973) diminish the magnitude of $\Delta$pH. However, quantitative estimates of $\Delta$pH under different conditions were required to provide critical tests of the chemiosmotic hypothesis Spinach chloroplast thylakoids appeared to be a good experimental subject for these investigations. Thylakoids catalyze rapid electron flow and phosphorylation and have very low ATPase activity. Rates of electron flow and phosphorylation may be very easily altered simply by changing the incident light intensity. More significantly, the $H^+$ electrochemical gradient may be more readily determined in thylakoids than in other phosphorylating organelles. Illuminated spinach chloroplast thylakoids suspended in media containing high $Cl^-$ concentrations appear to have only a slight electrical potential across them at the steady state (Rottenberg et al., 1972; Schröder et al., 1971). In contrast, the pH differential, which

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