Effects of the Mutation Glycine-222 → Aspartic Acid on the Functions of Elongation Factor Tu†

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ABSTRACT: We have studied the properties of a mutant elongation factor Tu, encoded by *tufB* (EF-TuBo), in which Gly-222 is replaced by Asp. For its purification from the kirromycin-resistant EF-Tu encoded by *tufA* (EF-TuAr), a method was developed by exploiting the different affinities to kirromycin of the two factors and the competition between kirromycin and elongation factor Ts (EF-Ts) for binding to EF-Tu. The resulting EF-TuBo·kirromycin and EF-TuAr·EF-Ts complexes are separated by chromatography on diethylaminoethyl-Sephadex A-50. For the first time we have succeeded in obtaining a *tufB* product in homogeneous form. Compared with wild-type EF-Tu, EF-TuBo displays essentially the same affinity for GDP and GTP, with only the dissociation rate of EF-Tu·GTP being slightly faster. Protection of aminoacyl-tRNA (aa-tRNA) against nonenzymatic deacylation by different EF-Tu species indicates that conformational alterations occur in the ternary complex EF-TuBo·GTP·aa-tRNA. However, the most dramatic modification is found in the EF-TuBo interaction with the ribosome. Its activity in poly(Phe) synthesis as well as in the GTPase activity associated with the interaction of its ternary complex with the ribosome·mRNA complex requires higher Mg^{2+} concentrations than wild-type EF-Tu (Mg^{2+} optimum at 10–14 vs. 6 mM), even if EF-TuBo can sustain enzymatic binding of aa-tRNA to ribosomes at low Mg^{2+}. The anomalous behavior of EF-TuBo is reflected in a remarkable increase of the fidelity in poly(Phe) synthesis, especially at high Mg^{2+} concentrations. Whereas the intrinsic GTPase (the activity found in the absence of any effector) of EF-TuBo is the same as for wild-type EF-Tu, aa-tRNA and ribosomes are incapable of stimulating the GTPase activity of EF-TuBo in the absence of mRNA. Kirromycin stimulates the GTPase of wild-type EF-Tu and EF-TuBo to the same extent with and without aa-tRNA, while ribosomes, normally the most important effectors of the reaction, are unable to enhance the EF-TuBo·kirromycin GTPase. These results indicate that the mutation Gly-222 → Asp causes an anomalous interaction of EF-TuBo with the ribosome in both the absence and the presence of aa-tRNA. This alteration increases the initial energetic constraints and makes the presence of mRNA and high Mg^{2+} concentration prerequisites for a productive interaction of the EF-TuBo ternary complex with the ribosome.

In the elongation cycle of protein biosynthesis, elongation factor Tu (EF-Tu)[1] mediates the binding of aa-tRNA to the ribosome·mRNA complex. Its activity is regulated by the nucleotides GTP and GDP. GTP allows EF-Tu to interact with aa-tRNA, forming a ternary complex that can bind to the ribosome following codon–anticodon interaction. The proper emplacement of aa-tRNA in the ribosomal A site is associated with the hydrolysis of GTP into GDP, inducing a conformational rearrangement of the factor to a form with lower affinity for aa-tRNA and the ribosome. As a consequence, EF-Tu·GDP dissociates from the ribosomal complex, allowing synthesis of a new peptide bond between aa-tRNA in the A site and peptidyl-tRNA in the P site [reviewed in Kaziro (1978), Bosch et al. (1983), and Parmeggiani and Swart (1985)]. The antibiotic kirromycin binds to EF-Tu in a 1:1 molar ratio, disrupting the EF-Tu functional cycle. It induces a conformation that recognizes aa-tRNA and the ribosome even in the presence of GDP, thus hindering the release of EF-Tu·GDP. As a consequence, polypeptide synthesis is inhibited and the ribosome is blocked on the mRNA (Parmeggiani & Sander, 1980; Parmeggiani & Swart, 1985).

Table I: List of Mutant EF-Tu Species

EF-Tu symbol[a]	mutation	behavior toward kirromycin	*E. coli* source
AsBs		sensitive	wild type (B)
D2216	Ala-375 → Val	resistant	D2216 (K12)
Ar	Ala-375 → Thr	resistant	PM455 (K12)
ArBo	Ar: Ala-375 → Thr Bo: Gly-222 → Asp	resistant	LBE2021 (K12)
Bo	Gly-222 → Asp	sensitive	LBE2021 (K12)

[a] A and B indicate that EF-Tu was derived from *tufA* and *tufB*, respectively. s indicates sensitive to kirromycin. r indicates resistant to kirromycin. o indicates mutated; sensitivity to kirromycin is recessive with respect to resistant EF-Tu.

Exceptionally for a bacterial protein, EF-Tu is encoded by two almost identical, distantly located genes *tufA* and *tufB*

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[1] Abbreviations: EF-Tu, elongation factor Tu; EF-Ts, elongation factor Ts; EF-G, elongation factor G; GDP, guanosine 5′-diphosphate; GTP, guanosine 5′-triphosphate; aa-tRNA, aminoacyl-tRNA; $tRNA^{Phe}$, tRNA isoaccepting Phe; mRNA, messenger RNA; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; DTT, dithiothreitol; TCA, trichloroacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; FPLC, fast-protein liquid chromatography.

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(Jaskunas et al., 1975). Since sensitivity to kirromycin is dominant over resistance due to the mode of action of the antibiotic, phenotypic expression of resistance requires alteration of both *tuf* genes [reviewed in Bosch et al. (1983), Parmeggiani and Sander (1980), and Parmeggiani and Swart (1985)]. Accordingly, mutant *Escherichia coli* strains have been isolated, harboring a *tufA* encoding an EF-Tu (EF-TuA) with a strongly reduced affinity for kirromycin and a *tufB* that either is inactive or encodes an EF-Tu, whose sensitivity to kirromycin is of a recessive nature (Table I). The first *tuf* gene combination is found in strain D2216, producing an EF-TuA that has Ala-375 replaced by Val (Fischer et al., 1977; Duisterwinkel et al., 1984). No *tufB* product could be detected in this strain. The second *tuf* gene combination is found in strain LBE2012 (Van de Klundert et al., 1977; Van der Meide et al., 1980). Interestingly, the kirromycin-resistant EF-TuA (designated EF-TuAr) from this strain also displays a substitution of Ala-375, in this case by Thr (Duisterwinkel et al., 1981a). In addition, *tufB* has undergone a mutation causing the replacement of Gly-222 by Asp (Duisterwinkel et al., 1984). Although the mutated EF-TuB (designated EF-TuBo) is sensitive to kirromycin in vitro, this factor is not blocked on the ribosome in the presence of the antibiotic. Thus its kirromycin sensitivity is recessive toward kirromycin resistance (Van der Meide et al., 1981; Duisterwinkel et al., 1981b).

The major difficulty in the purification of a *tuf* product carrying a mutation in a limited number of amino acid residues resides in its separation from the other *tuf* gene product. Here we describe a procedure that allows complete separation of EF-TuBo from EF-TuAr by exploiting their different affinities for kirromycin and the competition between EF-Ts and the antibiotic for binding to EF-Tu (Chinali et al., 1977). In our specific case, the difference between EF-TuAr and EF-TuBo concerns two mutated residues, Ala-375 → Thr and Gly-222 → Asp, and the C termini Gly and Ser, respectively (Arai et al., 1980). Whereas EF-TuAr has been isolated as a single gene product by inactivating *tufB* (Van der Meide et al., 1980, 1983), so far it has not been possible to isolate EF-TuBo from cells harboring an inactive *tufA*. Until now this situation has only permitted a partial purification of EF-TuBo from EF-TuAr (Van der Meide et al., 1981). The results of this work obtained with pure EF-TuBo show that the mutation Gly-222 → Asp strongly modifies the functional interaction between EF-Tu and the ribosome.

Materials and Methods

Adenosine 5′-triphosphate, GDP, GTP, phosphoenolpyruvate, and pyruvate kinase (EC 2.7.1.40) were purchased from Boehringer, Mannheim. [^{3}H]GDP and [^{14}C]Phe were from Amersham Int. plc, Amersham, England, and [^{3}H]Leu was from New England Nuclear. ^{32}P-Labeled inorganic phosphate was obtained from the Commissariat à l'Energie Atomique, Saclay, France, and [γ-^{32}P]GTP was prepared as described (Chinali et al., 1977). PMSF from Sigma was used as a freshly prepared 100 mM solution in dry 2-propanol. All other chemicals used were of reagent grade.

DEAE-Sephadex A-50 and the FPLC system with a MonoQ column were from Pharmacia Fine Chemicals. Kirromycin (mocimycin) was a kind gift from Dr. H. Beukers, Gist-Brocades, Delft, The Netherlands. Leucyl-tRNA synthetase was kindly donated by M. Fromant from the Laboratoire de Biochimie, Ecole Polytechnique.

Electrophoretically homogeneous, crystalline EF-Tu was isolated from wild-type *E. coli* B (EF-TuAsBs) and mutant *E. coli* K12 LBE2021 (EF-TuArBo; Van der Meide et al., 1983), by using essentially an already described procedure (Chinali et al., 1977). All other biological components not mentioned here were as previously reported (Chinali et al., 1977).

For technical details of the methods used for measuring poly(U)-directed poly(Phe) synthesis, misincorporation of Leu into poly(Phe), binding of GDP, and association and dissociation rate constants of EF-Tu·GTP and EF-Tu·GDP complexes, see legends to the figures. GTPase activiy was determined as liberation of inorganic phosphate as described by Parmeggiani and Sander (1981).

Buffers. Extensive dialysis (3–4 days) of EF-Tu·kirromycin in the presence of EF-Ts was against a buffer containing 25 mM Tris-HCl, pH 7.8, 1 mM EDTA, 50 mM NH_4Cl, 1 mM DTT, and 10% glycerol. Buffer A contained 50 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 80 mM KCl, and 7 mM 2-mercaptoethanol; when used in a salt gradient for DEAE-Sephadex A-50 chromatography, the KCl concentration was adapted. Buffer B contained 50 mM imidazoleacetate, pH 7.5, 50 mM KCl, 40 mM NH_4Cl and 7 mM 2-mercaptoethanol. Buffer C contained 50 mM imidazoleacetate, pH 7.5, 10 mM $MgCl_2$, 40 mM NH_4Cl, and 7 mM 2-mercaptoethanol.

Results

Isolation of Pure EF-TuBo. As a first approach to the separation of EF-TuAr and EF-TuBo, an attempt was made to exploit the differences in charge and isoelectric point resulting from the replacement of Gly-222 by Asp (these experiments are not illustrated). However, chromatography on DEAE-Sephadex A-50 or FPLC on MonoQ failed to resolve the mixture, indicating that the one-charge difference was insufficient for the purpose. Preparative chromatofocusing is also unsuccessful since both mutant species precipitate at their isoelectric points (EF-TuAr at pH 5.9 and EF-TuBo at pH 5.8). This precipitation leads to irreversible inactivation, even in the presence of excess GDP.

By exploiting their differential affinities for kirromycin and the fact that EF-Ts and kirromycin exclude each other in their binding to EF-Tu (Chinali et al., 1977), we could separate the two mutant factors. Addition of a critical amount of kirromycin to a mixture of equimolar amounts of EF-TuAr·EF-Ts, EF-TuBo·EF-Ts, EF-TuAr·GDP, and EF-TuBo·GDP results in the formation of EF-TuBo·kirromycin and the trapping of EF-TuAr in a stable complex with EF-Ts. The two complexes are readily separated by chromatography on DEAE-Sephadex A50, with EF-TuAr·EF-Ts being eluted at lower ionic strength than EF-TuBo·kirromycin (Figure 1). The concentrations of EF-TuAr, EF-TuBo, and EF-Ts in the mixture to be loaded on the column must be kept in the range between 5 and 10 μM, while the critical kirromycin concentration ranges from equimolar (to EF-TuBo) up to a 5-fold excess. At first, we started with a mixture of EF-TuAr·EF-Ts, EF-TuBo·EF-Ts, EF-TuAr·GDP, and EF-TuBo·GDP obtained by fractionation of the postribosomal supernatant (S-100) over DEAE-Sephadex A-50. Because of proteolytic phenomena in the following steps, later we used at least 70% pure EF-TuAr·EF-Ts and EF-TuBo·EF-Ts, supplemented with a mixture of once crystallized EF-TuAr·GDP and EF-TuBo·GDP (at least 95% pure). For a successful application of the procedure, the concentrations of EF-Tu and EF-Ts are critical. Too high concentrations of EF-Ts and too low concentrations of EF-Tu greatly reduce the yield of EF-TuBo·kirromycin. Indeed, the affinity of EF-Ts for EF-Tu is by 2 orders of magnitude greater than that of the antibiotic for wild-type EF-Tu (1 and 100 nM, respectively; Miller & Weissbach, 1971; Fasano et

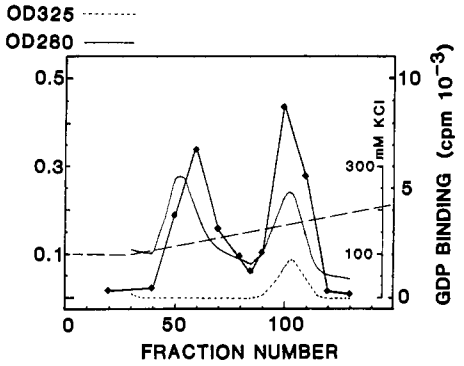

FIGURE 1: Separation of EF-TuAr·EF-Ts and EF-TuBo·kirromycin by chromatography over DEAE-Sephadex A-50. The mixture of EF-TuAr, EF-TuBo, EF-Ts, and kirromycin was preincubated in concentrations of 7.5, 7.5, 7.5, and 30 μM, respectively, in 30 mL of buffer A with 80 mM KCl and 1 mM PMSF for 30 min at 30 °C. The sample was applied to a DEAE-Sephadex A-50 column (50 × 0.9 cm) and eluted with a continuous gradient (buffer A, 100–250 mM KCl, and 1 μM kirromycin, total volume 600 mL). Absorbance at 280 nm (—), binding of [^{3}H]GDP (◆), and kirromycin absorbance at 325 nm (- - -) were determined.

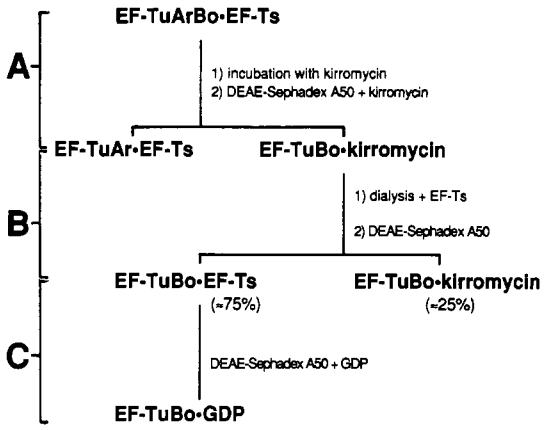

FIGURE 2: Schematic representation of the purification of EF-TuBo: (A) separation of EF-TuAr·EF-Ts and EF-TuBo·kirromycin; (B) removal of kirromycin from EF-TuBo; (C) isolation of EF-TuBo·GDP.

al., 1978; Parmeggiani & Swart, 1985). By application of the right concentrations of the different components, a complete separation of the two factors can be reproducibly obtained.

The EF-TuBo·kirromycin complex obtained from the chromatographic separation must be liberated from the antibiotic, which is partially achieved by extensive dialysis in the presence of EF-Ts. The resulting EF-TuBo·EF-Ts is completely purified from the remaining EF-TuBo·kirromycin by repeating the chromatography on DEAE-Sephadex A-50. The total yield of EF-TuBo is increased by dissociation of the persisting EF-TuBo·kirromycin in another dialysis step and subsequent chromatography. The final step of the procedure involves dissociation of the EF-TuBo·EF-Ts complex. After addition of a 10-fold excess of GDP, EF-TuBo·GDP and EF-Ts are separated by chromatography on DEAE-Sephadex A-50 in the presence of GDP. The steps of the purification procedure are outlined in Figure 2.

The purified EF-TuBo·GDP is homogeneous as judged by SDS/PAGE electrophoresis and isoelectrofocusing (Figure 3). In the beginning, the yield of pure EF-TuBo·GDP obtained by this method was rather low (10–20%). The recovery was considerably improved (up to 60–70%) by using enriched EF-Tu and EF-Ts fractions (see above) and by inhibiting protease activity with PMSF in all steps where kirromycin was present. It is known that kirromycin makes EF-Tu more susceptible to proteolytic cleavage (Douglass & Blumenthal,

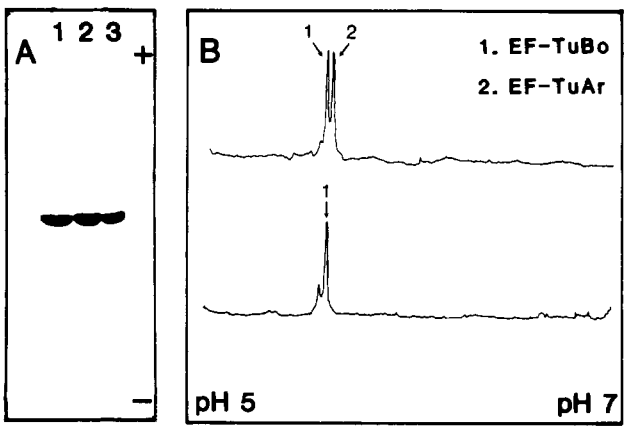

FIGURE 3: SDS/PAGE gel and isoelectrofocusing gel of isolated EF-TuBo. (A) Purity of EF-TuBo on 10% polyacrylamide denaturing gel. Five micrograms of EF-TuAsBs·GDP (1), EF-TuBo·GDP (2), and EF-TuBo·kirromycin (3) was applied in each lane. (B) Scanning profile of isoelectrofocusing gels of 10 μg of EF-TuArBo·GDP and 5 μg of EF-TuBo·GDP. The minor, acidic peak in the EF-TuBo gel is probably due to deamidation of the protein.

Table II: Apparent Dissociation Rate Constants k'_{-1} of Complexes between EF-TuBo or EF-TuAsBs and GDP/GTP, in the Absence and Presence of Kirromycin, Expressed as Half-Lives[a]

	EF-TuAsBs	EF-TuBo
EF-Tu + GDP	50 min	50 min
EF-Tu + GDP + 5 μM kirromycin	16 min	16 min
EF-Tu + GTP	71 s	51 s
EF-Tu + GTP + 5 μM kirromycin	52 min	52 min

[a] The reactions were carried out in buffer C by following the retention of the complexes on nitrocellulose filters, as previously described (Fasano et al., 1978). The apparent dissociation constants, K'_d, for complex formation between EF-Tu and GDP and GTP are 1 and 500 nM, respectively, in the absence of kirromycin. Kirromycin increases the EF-Tu affinity for GTP to K'_d values of 1 nM (Fasano et al., 1978).

1979). PMSF is added to a final concentration of 1 mM just prior to addition of the antibiotic to the mixture of EF-TuAr, EF-TuBo and EF-Ts, and again before addition of EF-Ts to EF-TuBo·kirromycin after the first chromatographic step.

The difficulties encountered in eliminating kirromycin from EF-Tu, even in the presence of EF-Ts, made us check the possibility of a photochemical cross-linking between antibiotic and factor, but no evidence was obtained for a covalent binding. Eccleston (1981) reports that dissociation of EF-Tu·kirromycin takes place with a half-life of 20 min at room temperature. The tight binding of kirromycin to EF-Tu observed in our experiments confirms this slow dissociation rate.

EF-TuBo Interacts with Guanine Nucleotides like Wild-Type EF-Tu. For a characterization of the interaction between EF-TuBo and GDP or GTP, the association and dissociation rate constants of the respective complexes were determined and compared with those of the wild-type factor (EF-TuAsBs). In Table II the half-lives of the different complexes are reported. No significant modification of the parameters is found with EF-TuBo·GDP. Neither is the association rate between EF-TuBo and GTP significantly changed. Only the dissociation rate of EF-TuBo·GTP is slightly enhanced, causing an increase (approximately 30%) of the K'_d value as already reported (Duisterwinkel et al., 1984). In the presence of kirromycin, we only studied the dissociation rates of EF-Tu·GDP and EF-Tu·GTP. Both mutant and wild-type complexes are affected in the same way by kirromycin: the dissociation rate of GDP from EF-Tu is enhanced, whereas that of GTP is greatly retarded (Fasano et al., 1978). These results

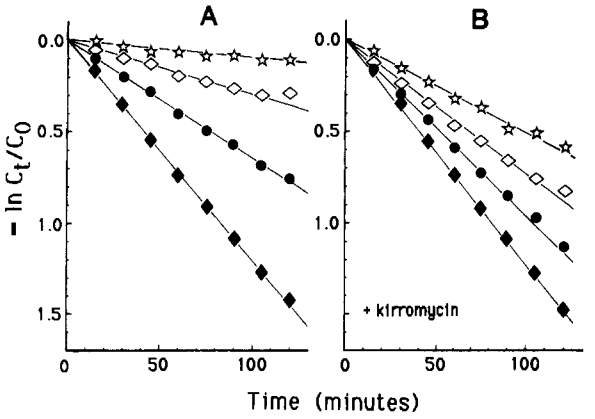

FIGURE 4: Hydrolysis protection of Phe-tRNA^{Phe} by EF-Tu·GTP in the absence (panel A) or presence of kirromycin (panel B). EF-Tu·GTP was present at a concentration of 0.8 μM, [^{14}C]Phe-tRNA^{Phe} (0.5 nmol of Phe/A_{260} unit; specific activity 1000 cpm/pmol) was 0.4 μM, and, if present, kirromycin was 30 μM. The assay was performed at 25 °C in a total volume of 100 μL; the sample volume was 10 μL. All other conditions were as described in Pingoud et al. (1977). Symbols: (☆) EF-TuAsBs, (◇) EF-TuBo, (●) EF-TuAr, and (◆) control (no EF-Tu).

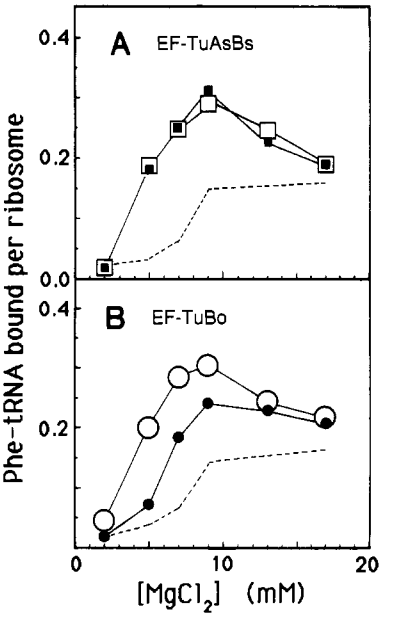

FIGURE 5: Enzymatic binding of Phe-tRNA^{Phe} to the A site of poly(U)-programmed ribosomes in the absence (open symbols) or presence (closed symbols) of kirromycin. The final solution (50 μL) contained ribosomes (0.2 μM), poly(U) (40 μg/mL), unacylated tRNA^{Phe} (0.4 μM; 0.5 nmol of Phe/A_{260} unit), GTP (0.1 mM), phosphoenolpyruvate (2 mM), pyruvate kinase (50 μg/mL), EF-Tu (0.3 μM), [^{14}C]Phe-tRNA^{Phe} (0.4 μM; 0.5 nmol of Phe/A_{260} unit; specific activity 1000 cmp/pmol), and, when present, 5 μM kirromycin in buffer B with the $MgCl_2$ concentration as indicated. Ribosomes, poly(U), and unacylated tRNA^{Phe} were preincubated at 10 mM Mg^{2+} and 30 °C for 15 min. EF-Tu·GDP was converted to EF-Tu·GTP during a preincubation at 30 °C for 15 min in the presence of GTP, phosphoenolpyruvate, and pyruvate kinase. At t = 0 min Phe-tRNA was added to the other components. The incubation was at 30 °C. After 5 min, samples of 40 μL were spotted on nitrocellulose filters and washed once with 3 mL of buffer. Panel A, EF-TuAsBs; panel B, EF-TuBo. The blank binding is indicated by the dashed line.

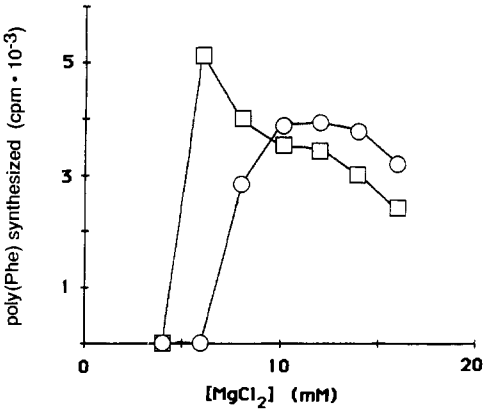

FIGURE 6: Mg^{2+} optimum of poly(Phe) synthesis of EF-TuAsBs (□) and EF-TuBo (○). Reaction mixtures (60 μL) contained GTP (2 mM), phosphoenolpyruvate (2.6 mM), pyruvate kinase (35 μg/mL), [^{14}C]Phe-tRNA^{Phe} (2 μM; 0.3 nmol/A_{260} unit; 100 cpm/pmol), EF-Tu (0.3 μM), EF-G (0.1 μM), ribosomes, (0.3 μM), and poly(U) (66 μg/mL) in buffer B with the $MgCl_2$ concentration as indicated. Incubation was at 37 °C for 10 min, and the reaction kinetics were still linear. Samples of 50 μL were applied to Whatman GF/A glass fiber filters and precipitated in 10% TCA, boiled in 5% TCA for 15 min, subsequently rinsed in 5% TCA, twice in ethanol/ether (1:1, v/v), and in ether, and dried, and the radioactivity was counted. Preincubation of the ribosomes with AcPhe-tRNA or addition of EF-Ts did not essentially change the obtained profiles.

suggest that the mutation Gly-222 → Asp does not essentially affect the interaction with GDP or GTP.

EF-TuBo Interacts Differently with aa-tRNA Relative to Wild-Type EF-Tu. The conformation of the ternary complex EF-Tu·GTP·aa-tRNA is of pivotal importance for the interaction between EF-Tu and the ribosome·mRNA complex. To obtain some clues on the effect of the mutation Gly222 → Asp, we have carried out protection experiments against nonenzymatic deacylation (Pingoud et al., 1977) with the different EF-Tu species. As shown in Figure 4A, protection by EF-TuBo is significantly less than that by EF-TuAsBs, with EF-TuAr being the least efficient as noted before (Van der Meide et al., 1983). Addition of kirromycin reduces protection by EF-Tu at all conditions tested (Figure 4B). This effect is more pronounced with EF-TuBo and EF-TuAsBs than with EF-TuAr.

Another criterion to examine the properties of the mutant ternary complex is the enzymatic binding of aa-tRNA to the ribosomal A site, as a function of Mg^{2+} concentration (Figure 5). Addition of kirromycin does not affect the enzymatic binding of aa-tRNA mediated by EF-TuAsBs, whereas that of EF-TuBo is greatly reduced, particularly at low Mg^{2+} concentrations. In the range of 2–5 mM Mg^{2+}, the inhibition is almost total (from 70 to 100%). These concentrations are usually considered to approach the intracellular values of Mg^{2+}.

EF-TuBo Does Not Sustain Poly(Phe) Synthesis at Mg^{2+} Concentrations Optimal for Wild-Type EF-Tu because of an Inefficient Triggering of Its GTPase upon Interaction with the Ribosome. Previously reported experiments carried out with a mixture of EF-TuAr and EF-TuBo in the absence of kirromycin (Van der Meide et al., 1981; Duisterwinkel et al., 1981b) did not reveal any consequence of the replacement of Gly-222 by Asp. The availability of pure EF-TuBo has made it possible to reinvestigate the activity of this EF-Tu mutant in poly(U) translation (Figure 6). EF-TuBo sustains poly(U)-directed poly(Phe) synthesis, but this activity requires 10–14 mM Mg^{2+}. At the Mg^{2+} concentrations optimal for wild-type EF-Tu-dependent poly(Phe) synthesis (about 6 mM) EF-TuBo is virtually inactive.

The clue for this misbehavior of EF-TuBo in poly(Phe) synthesis was found by monitoring EF-TuBo-dependent GTPase activity as a funtion of the Mg^{2+} concentration in a poly(U) translating system lacking EF-G. Since poly(Phe) synthesis can proceed, albeit slowly, in the absence of EF-G (Spirin, 1985), we studied translating ribosomes in the absence of the latter factor so that we could look at the GTPase activity

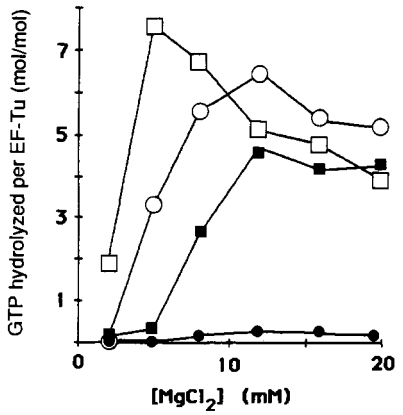

FIGURE 7: Mg^{2+} optimum of EF-Tu-dependent GTPase activity in poly(U) translating system lacking EF-G. Reaction mixtures in a final volume of 60 μL contained 0.1 μM EF-Tu, 0.1 μM EF-Ts, 0.17 μM ribosomes, 5.5 μM [γ-^{32}P]GTP, and no or 30 μg/mL poly(U) in buffer C with the $MgCl_2$ concentration as indicated. Incubation was for 6 min at 37 °C. Symbols: (□) EF-TuAsBs plus poly(U), (■) EF-TuAsBs minus poly(U), (○) EF-TuBo plus poly(U), and (●) EF-TuBo minus poly(U).

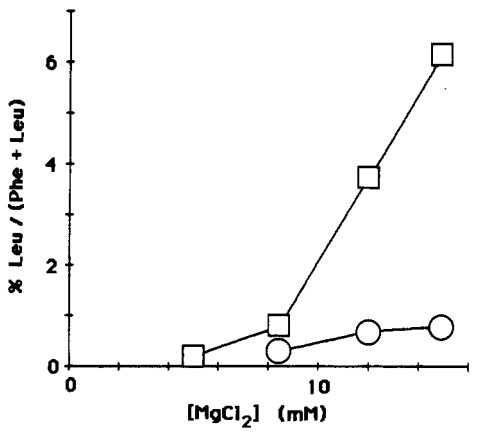

FIGURE 8: Fidelity of EF-TuAsBs (□) and EF-TuBo (○) in poly(U) translating system as a function of the Mg^{2+} concentration. Reaction mixtures in a final volume of 60 μL were as in Figure 6, except that [^{14}C]Phe-tRNA^{Phe} was replaced by 1 nmol of [^{14}C]Phe (10 cpm/pmol), 0.2 nmol of [^{3}H]Leu (2000 cpm/pmol), 25 μg of total tRNA, 1 mM adenosine triphosphate, and saturating amounts of both Phe-tRNA synthetase and Leu-tRNA synthetase. Incubation was for 20 min at 37 °C. Different ratios of EF-Tu vs. tRNA did not qualitatively change the presented results.

associated with EF-TuBo. As shown in Figure 7, the pattern of the GTPase activity resembles that found for phenylalanine incorporation: the optimum activity is shifted again to values between 10 and 14 mM Mg^{2+}. Strikingly, in the absence of poly(U) no GTPase activity is expressed, even at high Mg^{2+} concentrations, in contrast to the wild-type system. This result suggests that the GTPase activity of EF-TuBo is totally dependent on the presence of mRNA, i.e., on codon–anticodon interaction. It is germane to mention that the mutant factor can still sustain the enzymatic binding of aa-tRNA to the ribosome at Mg^{2+} concentrations of 2–6 mM, i.e., under conditions in which both GTPase and poly(Phe) synthesis are virtually abolished. However, the resulting complex is not very stable since we could not isolate it by gel filtration (label on the γ-phosphate of GTP; results not shown). These results strongly suggest an alteration of the EF-TuBo·GTP·aa-tRNA complex leading to an anomalous interaction with the mRNA·ribosome, unable to influence the EF-Tu GTPase center.

When we tested the fidelity of EF-TuBo in the poly(Phe) system by measuring the misincorporation of Leu, we observed a remarkable increase in accuracy (Figure 8). The effect becomes progressively evident with increasing Mg^{2+} concentrations, a condition that is known to reduce the specific requirements for the interaction between EF-Tu and the ribosome (Jelenc & Kurland, 1979). This result complements the observation of Figure 7, showing that a productive interaction between EF-TuBo·GTP·aa-tRNA and the ribosome leading to GTP hydrolysis requires codon–anticodon interaction.

EF-TuBo Diplays a Normal Intrinsic GTPase. The action of EF-TuBo in protein synthesis in anomalous: it can sustain peptide bond formation only in the presence of elevated Mg^{2+} levels. Our results suggest that this defect is associated with a deficient activation by the ribosome of the EF-Tu GTPase center at low Mg^{2+}. This interpretation is supported by investigating the behavior of the intrinsic GTPase of EF-TuBo and wild-type EF-Tu, that is, the activity obtained with the factor alone in the absence of any other effector. Under these conditions GTP hydrolysis occurs at a low rate and its turnover is inhibited by the product GDP, which has a much higher binder affinity than GTP (Figure 9). As shown before (Fasano et al., 1982b; Swart et al., 1982), this activity is stimulated by the addition of monovalent cations. At low monovalent cation concentrations the catalytic center of the mutant factor is more active than that of wild type, but this difference disappears with increasing salt concentrations. We conclude that the basic catalytic activity of EF-TuBo and that of wild-type EF-Tu are very much alike. This shows that the catalytic center of the mutant is not directly involved in the deficient activation of the GTPase reaction during poly(U) translation.

Ribosomes Cannot Stimulate the GTPase of EF-TuBo·Kirromycin. The antibiotic kirromycin activates the GTPase activity of wild-type EF-Tu by enhancing the catalytic reaction and the exchange of bound GDP with free GTP (Wolf et al., 1974; Fasano et al., 1978). A 2-fold stimulation of the hydrolysis reaction is observed upon addition of saturating amounts of aminoacyl-tRNA to EF-Tu·kirromycin. Ribosomes, alone or together with aminoacyl-tRNA, have a much stronger stimulatory effect [Figure 10A; as observed previously by Bocchini et al. (1980)]. With EF-TuBo, the basal activity induced by kirromycin and the stimulation by aa-tRNA are exactly the same as for wild type (Figure 10B) regardless of the Mg^{2+} concentration (not illustrated). The addition of ribosomes [poly(U) programmed or not], strongly stimulating the wild type, does not exert any influence on the mutated factor. This shows that the interaction between ribosomes and either EF-TuBo·GTP·kirromycin or its quaternary complex with aa-tRNA is the altered step. The lack of a ribosomal effect complements our observation that in the absence of kirromycin the activation of the mutant's GTPase center upon interaction with the ribosome requires codon–anticodon interaction. Moreover, it appears that the additional changes induced by the antibiotic make the conformation of the quaternary complex EF-TuBo·kirromycin·GTP·aa-tRNA so distorted that even addition of poly(U) does not stimulate a productive interaction with the ribosome.

Discussion

In this work we describe a method for the separation of two mutant EF-Tu species, EF-TuAr and EF-TuBo, from each other on the basis of their different affinities for kirromycin and the competition between the antibiotic and EF-Ts for binding to EF-Tu. This procedure can be applied to the isolation of any kind of mutant factors regardless of charge differences and therefore represents a method of general use

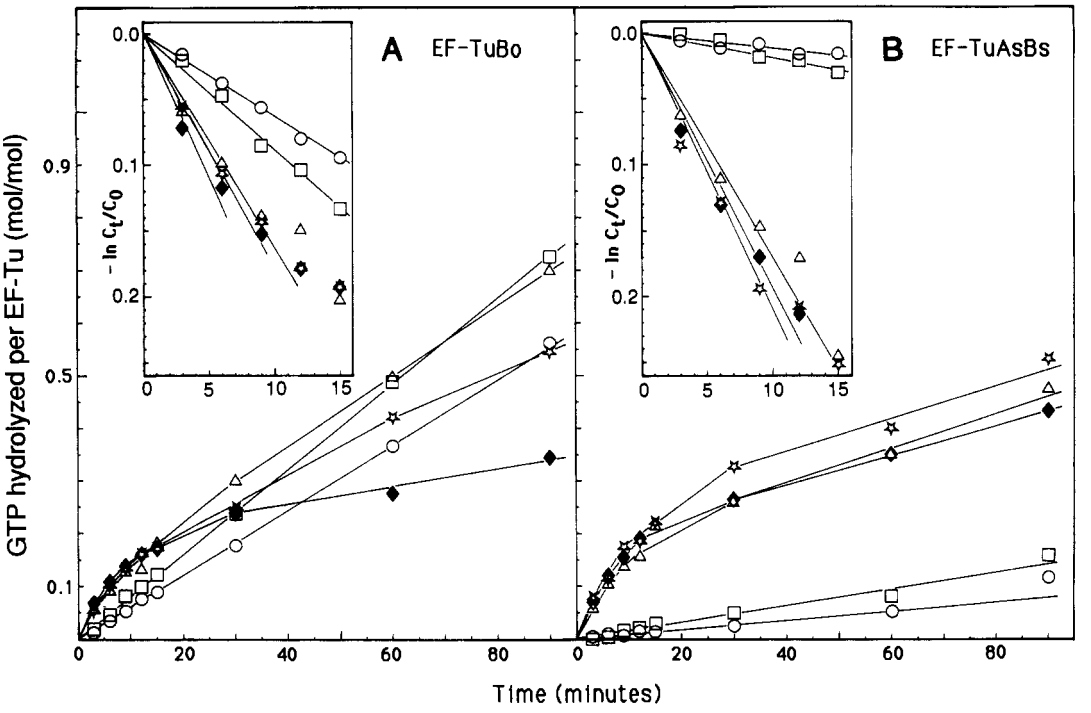

FIGURE 9: Intrinsic GTPase activity of EF-Tu·GTP. EF-Tu was separated from bound GDP as described (Fasano et al., 1982a). To allow formation of the complex EF-Tu·GTP, nucleotide-free EF-Tu and [γ-^{32}P]GTP (specific activity 10^4 cpm/pmol) were incubated at 0 °C for 15 min in buffer C without NH_4Cl. Then the NH_4Cl concentration was adjusted to the desired value: 0 (○), 40 (□), 200 (△), 400 (☆), or 800 mM (♦). Final concentrations of EF-Tu and GTP were 0.4 and 1.5 μM, respectively. The reaction was started by rapidly warming the solution to 37 °C, at the indicated times 10-μL samples were taken from the original 100-μL volume, and the reaction was stopped in 10 μL of 1 N $HClO_4$ containing 1 mM KH_2PO_4. Semilogarithmic plots for the initial breakdown of the EF-Tu·GTP complex are shown in the insets. Panel A, EF-TuBo; panel B, EF-TuAsBs.

applicable for the separation of mutated, plasmid-borne *tuf* products from the host cell factor (our unpublished results). The prerequisite is the use of host cells containing either kirromycin-resistant EF-Tu or kirromycin-sensitive EF-Tu (Van der Meide et al., 1983), depending on the respective kirromycin sensitivity or resistance of the mutant factor to be purified. It should be emphasized that this method allows the isolation of mutant factors unable to sustain cell growth, which therefore requires the additional presence of a fully active EF-Tu.

The homogeneous EF-TuBo appears to possess some interesting properties. Its interaction with the ribosome is unusual, whereas at first sight, binding of GDP, GTP, and aa-tRNA is not dramatically changed. However, at low Mg^{2+} concentrations enzymatic binding of aa-tRNA to the ribosome is unaccompanied by GTP hydrolysis and subsequent peptide bond formation; elevated Mg^{2+} levels are needed to restore these reactions, but GTP hydrolysis remains totally dependent on codon–anticodon interaction. From this it becomes clear that the minor quantitative differences found by testing the EF-TuBo·GTP complex with aa-tRNA are not representative of the functional effects of the conformational changes introduced by the mutation. The importance of these alterations becomes apparent when the interaction between EF-Tu in ternary complex and ribosomes, normally leading to GTP hydrolysis and peptide bond formation, is examined.

The absence of GTP hydrolysis is not an endogenous property of the mutant factor, as can be seen from the normal activity of the intrinsic GTPase in both the absence and presence of kirromycin. Our results strongly suggest that the main functional consequence of the mutation resides in the inability of ribosomes to activate efficiently the catalytic center of EF-TuBo. An anomalous conformation of the factor hinders its productive interaction with the ribosome, and consequently, at low Mg^{2+} concentrations the absence of GTP hydrolysis prevents the correct positioning of aa-tRNA in the ribosomal acceptor site and peptide bond formation.

Another probable consequence of an anomalous conformation of the mutant ternary complex is the very accurate translation of the messenger at elevated Mg^{2+} levels. At low Mg^{2+} concentrations (approaching intracellular values) or in polymix (Jelenc & Kurland, 1979), wild-type EF-Tu can sustain accurate polypeptide synthesis in vitro. These conditions are too restrictive for the mutant factor, and virtually no activity is observed [this paper and Tapio and Kurland (1986)]. When the Mg^{2+} concentration is raised, the constraint imposed on the wild-type system to perform accurate protein synthesis is partially relieved and miscoding increases progressively. Under these less restrictive conditions, the interaction between EF-TuBo·GTP·aa-tRNA and the programmed ribosome becomes productive, leading to GTP hydrolysis and peptide bond formation, but miscoding remains at a very low level. The speed of the initial selection step for recognition of the proper ternary complex is probably slowed down by the inefficient induction of the EF-TuBo center for GTP hydrolysis, increasing the accuracy. In agreement with this possibility, an increase of the Mg^{2+} concentration has recently been shown to accelerate hydrolysis of GTP on the ribosome and to lower the rate of dissociation of the ternary complex EF-Tu·GTP·aa-tRNA from the ribosome (Karim & Thompson, 1986).

Kirromycin inhibits polypeptide synthesis in vitro sustained by EF-TuBo (Duisterwinkel et al., 1984; Swart et al., 1984). Clearly, the interaction between the ternary complex EF-TuBo·GTP·aa-tRNA and the ribosome is already defective and kirromycin induces an additional alteration. As a consequence, the mutant complex cannot overcome the initial constraints conditioning a stable binding to the ribosome. This can be derived from two observations made in the presence of the antibiotic: enzymatic binding of aa-tRNA to the A site

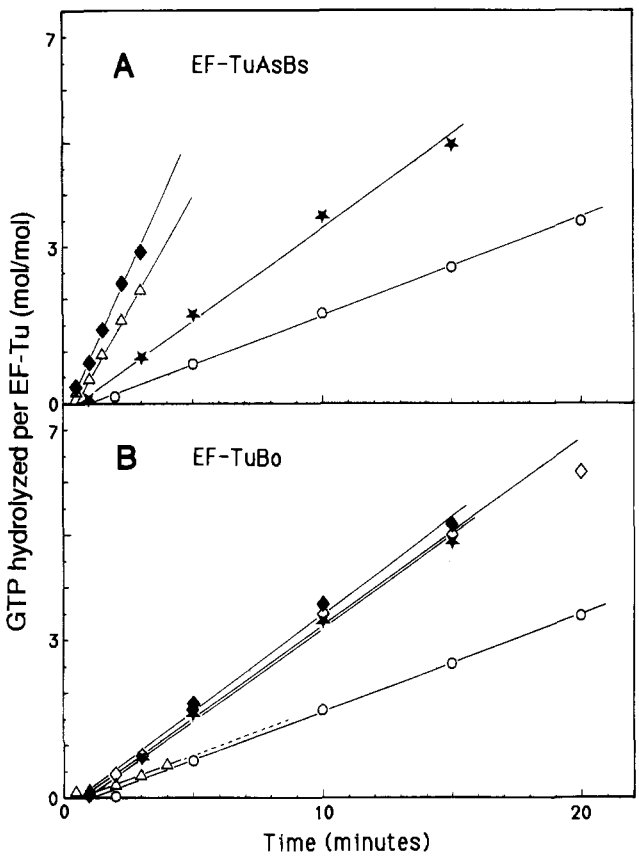

FIGURE 10: GTPase activity of EF-Tu·GTP·kirromycin. Reaction mixtures (total volume 70 μL) contained 0.4 μM EF-Tu·GDP, 50 μM kirromycin, and 12 μM [γ-^{32}P]GTP (specific activity 2400 cpm/pmole in buffer C with 100 mM NH_4Cl. The incubation was at 30 °C, and the reaction was started upon addition of GTP. At the indicated times 10-μL samples were taken and processed as described in the legend to Figure 9. Panel A, EF-TuAsBs; panel B, EF-TuBo. Symbols: (○) EF-Tu·kirromycin, (★) EF-Tu·kirromycin + 4 μM Phe-tRNA^{Phe} (0.3 nmol of Phe/A_{260} unit of tRNA), (△) EF-Tu·kirromycin + 1.2 μM ribosomes, (◆) EF-Tu·kirromycin + 4 μM Phe-tRNA^{Phe} + 1.2 μM ribosomes, and (◇) EF-Tu·kirromycin + 4 μM Phe-tRNA^{Phe} + 1.2 μM ribosomes + 12 μg/mL poly(U).

is decreased at low Mg^{2+} concentrations, and the GTP hydrolysis is not triggered by the ribosome.

The altered interaction between EF-TuBo and the ribosome is probably caused by the mutational event replacing Gly-222 by Asp. Nevertheless, we cannot exclude that some of the described functional effects of EF-TuBo could be typical for the homogeneous, wild-type *tufB* product; the functional effect of the different C termini of the two *tuf* products has yet to be studied in detail. X-ray diffraction studies of mildly trypsinized EF-Tu·GDP have located Gly-222, contrary to an earlier report (Duisterwinkel et al., 1984), in the middle domain comprising the amino acid residues 200–296 (Jurnak, 1985; La Cour et al., 1985; Parmeggiani et al., 1987). The three-dimensional structure of the middle domain is still too poorly defined to permit any conclusions concerning the site of the mutation and the defects of the mutant factor EF-TuBo. It can only be said that the functional changes observed in the three mutated factors isolated until now are probably all the results of long-range effects. This interpretation is supported by the finding that the two kirromycin-resistant EF-Tu mutants EF-Tu$_{Ala375→Val}$ and EF-Tu$_{Ala-375→Thr}$ reveal specific changes in the interaction with GTP and aa-tRNA as well as in their basic GTPase activity independent of aa-tRNA, ribosomes, and kirromycin (intrinsic GTPase activity; Fasano & Parmeggiani, 1981; Ivell et al., 1981; Swart et al., 1982; Sam et al., 1985).

Finally, the data presented in this paper strongly suggest that EF-TuBo is unable to sustain polypeptide synthesis by its own at near-physiological Mg^{2+} concentrations. Strains producing EF-TuBo as the sole *tuf* gene product may thus be expected to be nonviable, explaining why all *E. coli* strains known to produce EF-TuBo contain an active *tufA* product. However, this does not mean that EF-TuBo is nonfunctional in cells harboring an active *tufA* gene. Vijgenboom et al. (1985) recently demonstrated that EF-TuBo amplifies the nonsense suppressor activity of EF-TuAr, resulting in a severalfold higher suppression than in the case of EF-TuAr alone. It was concluded that the two mutant factor complement each other in vivo in the suppression of nonsense codons, which means that they cooperate on the ribosome during translation. The availability of homogeneous EF-TuBo has enabled us to study the question in vitro. The results obtained so far and to be published elsewhere suggest that EF-TuAr and EF-TuBo may display cooperativity also in vitro, both on and outside of the ribosome.

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Registry No. GTP, 86-01-1; GDP, 146-91-8; GTPase, 9059-32-9; Mg, 7439-95-4; kirromycin, 50935-71-2.

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Covalent Aspartylation of Aspartyl-tRNA Synthetase from Bakers' Yeast by Its Cognate Aspartyl Adenylate: Identification of the Labeled Residues†

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ABSTRACT: Aspartyl-tRNA synthetase from bakers' yeast gives an unstable complex with the cognate adenylate, which reacts after dissociation with amino acid side chains of the protein. This leads to a covalent incorporation of aspartic acid into aspartyl-tRNA synthetase via amide or ester bonds formed between the α-carboxyl group of activated aspartic acid and accessible lysines, serines, and threonines. This property is used to label the peptides at the surface of the enzyme. The main labeled residues have been identified, and their location in the primary structure is discussed in relation to structural properties of aspartyl-tRNA synthetase.

In a previous paper (Kern et al., 1985) a peculiar property of aspartyl-tRNA synthetase from bakers' yeast was reported: in the absence of $tRNA^{Asp}$ the enzyme forms a weak complex with its cognate aspartyl-adenylate, which easily dissociates from the synthetase. The activated carboxyl group of aspartic acid will then react with any nucleophilic acceptor present in the reaction mixture, in particular with amino acid side chains of the enzyme itself. By use of a chemical labeling technique it was shown that this phenomenon led to covalent incorporation of aspartic acid residues into the protein via the formation of stable amide bonds with lysine residues, their number depending not only on the pH of the reaction mixture but also on the chemical nature of the buffer chosen. Furthermore, this covalent binding of as many as eight aspartates per enzyme subunit did not affect the maximal velocity of the overall aminoacylation of $tRNA^{Asp}$ but significantly reduced the affinity for this tRNA. This indicates that some of the aspartylated residues must play a part, directly or indirectly, in tRNA binding. Finally as discussed in this initial study, aspartylation of aspartyl-tRNA synthetase probably occurs in accessible regions of the protein so that the in situ synthesized aspartyl adenylate can be used as a structural probe to map the surface of this protein. This is of particular interest as the determination of the crystallographic structures of both aspartyl-tRNA synthetase and its complex with $tRNA^{Asp}$ is well under way (Dietrich et al., 1980; Lorber et al., 1983a; Moras et al., 1983).

In this paper we bring new data concerning the aspartylated residues: indeed, isolation and sequencing of the labeled peptides definitely establish that the activated α-carboxyl group of aspartic acid not only reacts with lysines but also forms

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