Mapping the Effector Region in *Thermus thermophilus* Elongation Factor Tu†

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ABSTRACT: Native elongation factor Tu from *Thermus thermophilus* is initially attacked by various endoproteases in a region spanning amino acid residues 40–70. By comparing the hydrolysis rates of nucleotide-free and GDP-bound EF-Tu, only a small difference was observed for the tryptic cleavage at Arg-59. Protease V-8 attacks Glu-55 only in a GDP/GTP form, whereas this enzyme exclusively hydrolyze Asn-64 in nucleotide-free EF-Tu, even when the protein had been previously cleaved at Arg-59. Binding of GDP leads to a 42-fold decreased rate of hydrolysis by the Lys-C protease at Lys-52. It also reduces the accessibility of Lys-275 to trypsin, reflecting a "long-range" effect from nucleotide binding domain I to domain II. Only slight differences were observed in the rate of hydrolysis at all positions in the GDP- versus the GTP-bound form. The intrinsic GTPase activity was slightly reduced in trypsin-treated EF-Tu, significantly impaired in EF-Tu cleaved at Lys-52, and completely abolished in EF-Tu cleaved at Asn-64. No ribosome-induced GTPase activity was observed for protease-cleaved EF-Tu's. Treatment of these proteins with periodate-oxidized GDP or GTP followed by cyanoborohydride led to covalent modification of the new N-terminus located exclusively within region 52–60. The highest reactivity was shown by the N-terminus of Glu-56. Additionally, lysine residues in the native protein sensitive to affinity labeling [Peter, M. E., Wittmann-Liebold, B., & Sprinzl, M. (1988) *Biochemistry 27*, 9132–9139] lost their reactivity upon cleavage of EF-Tu in region 52–60, suggesting an altered structure of the cleaved protein. We conclude that region 52–60 is modulating the nucleotide binding site structure and is involved in the intramolecular signal transduction for the GTPase activation.

Elongation factor Tu (EF-Tu)[1] promotes the binding of aminoacyl-tRNA (aa-tRNA) to ribosomes during polypeptide chain elongation. In this process, EF-Tu interacts sequentially with GTP, aa-tRNA, ribosomes, GDP, and EF-Ts (Miller & Weissbach, 1977). EF-Tu is a member of a family of guanine nucleotide binding proteins which includes translational factors (Kaziro, 1978), signal transducing proteins (Gilman, 1987), and the ras gene family (Barbacid, 1987). The GTP binding proteins function by switching between a GTP-bound "signal on" activating conformation and a GDP-bound "signal off" latent conformation.

Tryptic excision of the peptide Asp-45–Arg-58 has facilitated the crystallization of *Escherichia coli* EF-Tu (Jurnak, 1985; la Cour et al., 1985). The β-strand b in the three-dimensional structure corresponds to an area of $p21^{ras}$ implicated in effector interaction (Sigal et al., 1986). The two published EF-Tu structures are not in agreement over the location of β-strand b. In the model of la Cour et al., it runs at its C-terminus antiparallel to the adjacent β-strand c, and its N-terminus is folded back, directed toward domain II. In the model of Jurnak, β-strand b is running antiparallel, side by side over the whole 10 amino acid residues to β-strand c. We now present results which are in favor of the model of Jurnak. However, the location of the loop region of amino acids 39–58 remains obscure in both models as well as its function. Nevertheless, the proposed functional models for the α-subunits of the eucaryotic G-proteins (Masters et al., 1986), transducin (Hingorani & Ho, 1987), procaryotic initiation factor 2 (Cenantiempo et al., 1987), and GTP binding proteins in general (Bourne, 1986) are based on the incomplete crystal structure of the *E. coli* EF-Tu·GDP G-domain (Jurnak, 1985; la Cour et al., 1985).

One of the methods to probe protein structures is to examine the susceptibility of certain amino acid residues to proteolytic enzymes. This approach has been elucidated in the case of EF-Tu from *E. coli* (Jacobson & Rosenbusch, 1977; Douglass & Blumenthal, 1979; Wittinghofer et al., 1980), eEF-Tu (Slobin et al., 1981, Möller et al., 1987; Miyazaky et al., 1988), and EF-2 (Nilsson & Nygård, 1988), where it was shown that trypsin rapidly cleaves the factors at a limited number of sites to yield a core fragment retaining some functional properties of the intact enzyme. We have now combined this method with affinity labeling of the GDP/GTP binding site to study the structure of EF-Tu. EF-Tu from *Thermus thermophilus* can be specifically modified at Lys-52 by both GDP_{oxi} and GTP_{oxi} (Peter et al., 1988). This demonstrates that this residue is in the vicinity of the bound nucleotide although it is not part of one of the known consensus sequences for nucleotide binding in GTP binding proteins (Dever et al., 1987).

In this report, we present studies on the cleavage of EF-Tu from *T. thermophilus* with different endoproteases in the region, which is assumed to play a functional role in the binding of effector molecules. The reactivity of a variety of such modified proteins in the affinity labeling reaction with periodate-oxidized GDP or GTP as well as their GTPase activity was examined.

Materials and Methods

[U-^{14}C]GTP (575 mCi/mmol) and [γ-^{32}P]GTP (10 Ci/mmol) were purchased from Amersham-Buchler (Braunschweig, FRG). [^{32}P]Orthophosphate (9000 Ci/mmol) was obtained from Du Pont/New England Nuclear (Bad Nauheim, FRG). [β-^{32}P]GDP (100 mCi/mmol) was prepared as described by Johnson and Walseth (1979). $NaIO_4$, NaCN-

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[1] Abbreviations: EF-Tu and EF-Ts, elongation factors Tu and Ts, respectively; eEF-Tu, eukaryotic EF-Tu; $EF\text{-}Tu_{Xn}$, EF-Tu specifically cleaved at amino acid residue X (in one-letter code) at position *n* in the *T. thermophilus* sequence; aa-tRNA, aminoacyl transfer RNA; HPLC, high-performance liquid chromatography.

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BH_3, and $NaBH_4$ were from Serva (Heidelberg, FRG). Acrylamide and *N,N'*-methylenebis(acrylamide) were obtained from BRL (Eggenstein, FRG). TPCK-treated trypsin and α-chymotrypsin were from Worthington (Freehold, NJ); the submaxillary protease (250 units/mg) was purchased from Pierce (Rodgau, FRG), the *Lysobacter enzymogenes* Lys-C protease (30 units/mg) from Boehringer (Mannheim, FRG), and the *Staphylococcus aureus* V-8 protease (500 units/mg) from Miles (Naperville, IL). EF-Tu·GDP was purified from *T. thermophilus* cells, strain HB8, by using a modified method described by Leberman et al. (1980). Anion-exchange chromatography on DEAE-Sepharose CL-6B (Pharmacia, Uppsala, Sweden) and gel permeation chromatography on AcA-44 (LKB, Bromma, Sweden) have been replaced by the respective chromatographies using Q-Sepharose Fast Flow and Sephacryl S-200 HR, both obtained from Pharmacia. The dialysis step between both separations could be avoided by dissolving the ammonium sulfate precipitate in a minimal volume of buffer and applying the solution directly to the gel permeation column. Nucleotide-free EF-Tu (21 000 units/mg) was prepared as previously described (Seidler et al., 1987). *E. coli* 70S ribosomes were isolated as described elsewhere (Gavrilova & Spirin, 1975).

Probing of EF-Tu with Different Endoproteases. Digestions with endoproteases were done with 20 μM nucleotide-free EF-Tu in a total volume of 100 μL containing 50 mM KCl, 10 mM $MgCl_2$, and 50 mM borate, pH 7.5 (buffer A), and, if required, 50 μM GMP, GDP, or GTP. At various times, 10-μL aliquots of the digestion mixture were withdrawn, mixed with 10 μL of sample buffer, and boiled for 2 min. The fragments were separated by 15% SDS-PAGE according to Laemmli (1970). The lanes were scanned with a laser densitometer (Model Ultroscan XL, LKB). The GDP binding activities of the modified proteins were determined by the nitrocellulose filter binding assay (Arai et al., 1972).

Determination of Cleavage Sites of Different Endoproteases on EF-Tu. EF-Tu was digested with either TPCK-treated trypsin, Lys-C protease, or *S. aureus* protease V-8 in buffer A. The resulting fragments were isolated by gel permeation chromatography on Superose 12 HR 10/30 (Pharmacia) or by reversed-phase chromatography on either Vydac C_4/300 A ("The Separation Group", Hesperia, CA) or LiChrospher RP-18 (Merck, Darmstadt, FRG) and sequenced on a gas-phase sequencer as previously described (Peter et al., 1988).

Testing the GTPase Activity of EF-Tu. The reaction mixture contained 150 mM NH_4Cl, 50 mM KCl, 20 mM $MgCl_2$, 50 mM Tris/HCl, pH 7.6, 10 μM [γ-^{32}P]GTP (8500 cpm·pmol^{-1}), and in some cases 10 μM *E. coli* 70S ribosomes in a total volume of 80 μL. The reaction was started by the addition of 10 μM EF-Tu at 37 °C. Liberation of P_i was followed by chromatographic analysis of 1 μL of the reaction mixture withdrawn at different times on PEI–cellulose sheets. The ascending chromatography was developed in 1 M formic acid and 0.8 M LiCl. Each lane was scanned with a Berthold thin-layer scanner II.

Affinity labeling of EF-Tu with periodate-oxidized nucleotides was performed as previously published (Peter et al., 1988). Limited cleavage of the polypeptide chain with a specific endoprotease prior to labeling was performed as follows: 20 μg of EF-Tu·nucleotide complex (EF-Tu/nucleotide = ratio 2/1) in buffer A was alternatively incubated with 0.1 μg of trypsin for 15 min, 10 μg of submaxillary protease for 60 min, 5 μg of Lys-C protease for 60 min, 0.5 μg of V-8 protease for 20 h, or 20 μg of chymotrypsin for 30 min. All incubations were carried out at 37 °C.

Table I: Initial Cleavage Sites for Different Endoproteases in *T. thermophilus* EF-Tu

protease	mol wt of isolated fragments (×10^{-3})[a]	N-terminal sequence	cleavage site	positions in sequence
trypsin	(1) 39.8	GITIN TAHVE	Arg-59–	Gly-60–Glu-405
	(2) 13.9	TLQ	Lys-275–	Thr-276–Glu-405
Lys-C protease	(1) 41.6	APEER	Lys-52–	Ala-53–Glu-405
	(2) 39.8	GITIN	Arg-59–	Gly-60–Glu-405
	(3) 25.9	APEER ARGIT INTAH V	Lys-45–	Asp-46–Lys-275
	(4) 23.8	R/YS/ VD/P	Lys-74–	Arg-75–Lys-275
V-8 protease	(1) 45.8	FVRTK PHVNV GTI	Glu-4–	Phe-5–Glu-405
	(2) 40.7	ERARG ITINT AH	Glu-55–	Glu-56–Glu-405
	(3) 38.7	TAHV/ Y/	Asn-64–	Thr-65–Glu-405
	(4) 37.6	TAKRH YS	Glu-71–	Thr-72–Glu-405
	(5) 23.2	YIPTP VR	Glu-208–	Tyr-209–Glu-405

[a] The apparent molecular weights of the fragments were determined by 15% SDS-PAGE.

Analysis of Affinity-Labeled EF-Tu. After being labeled, the protein was analyzed by adding trypsin to a concentration of 50 μg/mL. The solution was frozen and lyophilized following incubation at 37 °C for 30 s or 30 min. Analysis of labeled tryptic fragments on a 12.5% SDS–urea–PAGE system was done as described (Peter et al., 1988) with the following modifications: 2 M urea and 0.33% *N,N'*-methylene(bisacrylamide) were used for the gel.

Determination of the Modification Site in Trypsin-Cleaved EF-Tu. Affinity labeling of 500 μg of trypsin-treated EF-Tu with [U-^{14}C]GTP_{oxi} or [U-^{14}C]GDP_{oxi}, cleavage with cyanogen bromide, separation of labeled peptides by gel permeation chromatography, and also separation of the resulting labeled peptide pool by reversed-phase HPLC were all done as previously described (Peter et al., 1988). The purified labeled peptide was subjected to 10 cycles of Edman degradation in a liquid-phase sequencer (Model 810, Knauer, Berlin, FRG) by fixing the peptide on a poly(vinylidene difluoride) membrane using purified polybrene. The remaining labeled peptide was hydrolyzed on the filter for 20 h at 115 °C in 5.7 M HCl containing 0.02% β-mercaptoethanol and 0.001% phenol after evaporation and flushing with nitrogen. The amino acid composition of the peptide hydrolysate was determined by the automated *o*-phthaldialdehyde precolumn derivatization method (Ashman & Basserhoff, 1985).

Results

Influence of the Bound Nucleotide on EF-Tu Structure. Treatment of EF-Tu from *T. thermophilus* with different endoproteases leads to an initial cleavage in a region spanning amino acid residues 40–70 located within domain I (residues 1–211). Secondary cleavage sites are located in domain II (residues 212–311) and in the hinge region between domains I and II. Fragments generated under various conditions were isolated and sequenced (Table I). For measurement of the rate of hydrolysis in the presence and/or absence of nucleotide, we prepared a nucleotide-free EF-Tu which was then treated with GDP. In control experiments, GMP, which does not bind to the active site of EF-Tu (Arai et al., 1978), was added to the reaction mixture (Figure 1).

The kinetics of the hydrolysis of a respective amino acid residue are generally slower in the nucleotide-bound state of the protein than in the apoprotein (Figure 1). After trypsin initially cleaved the Arg-59/Gly-60 peptide bond, the rate of hydrolysis was 1.6-fold higher in the presence of GMP versus GDP. On the other hand, EF-Tu·GDP was 42 times more resistant against treatment with the Lys-C protease (Figure

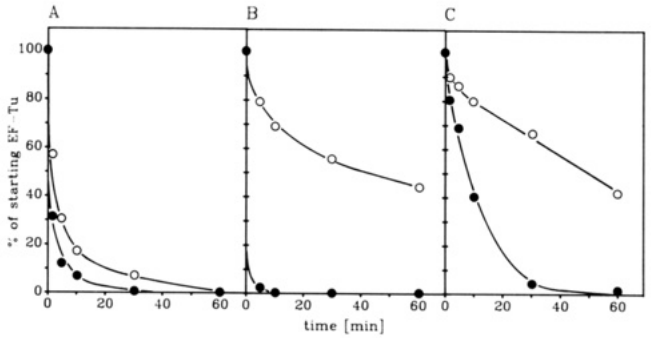

FIGURE 1: Cleavage of EF-Tu by endoproteases. Nucleotide-free EF-Tu was digested with trypsin (enzyme/substrate ratio 1/2000, w/w) (A), Lys-C protease (ratio 1/4) (B), or *S. aureus* V-8 protease (ratio 1/5) (C) in the presence of GMP (●) or GDP (○) at 37 °C as described under Materials and Methods. The fragments were quantified by scanning of SDS–PAGE gels.

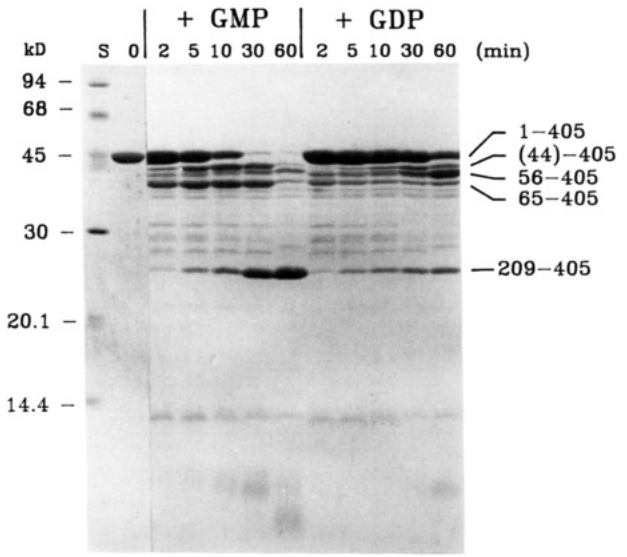

FIGURE 2: Effect of GDP on the cleavage of EF-Tu by *S. aureus* V-8 protease. Nucleotide-free EF-Tu was incubated with V-8 protease (ratio 1/5) in the presence of GMP (lanes 3–7) or GDP (lanes 8–12) at 37 °C. At the indicated times, 10-μL aliquots were analyzed by 15% SDS–PAGE. Lane S, standard proteins (Pharmacia).

1B) than nucleotide-free EF-Tu. This enzyme leads to hydrolysis of the Lys-52/Ala-53 peptide bond. In both cases, EF-Tu did not lose its ability to bind GDP during the process of cleavage at the first site (see below).

The V-8 protease hydrolyzed nucleotide-free EF-Tu 8.7 times faster than EF-Tu·GDP (Figure 1C). In this case, the peptide bond Asn-64/Thr-65 was cleaved in the apoprotein and the peptide bond Glu-55/Glu-56 in EF-Tu·GDP (Figure 2). During this cleavage of nucleotide-free EF-Tu at 37 °C, another peptide bond, probably Glu-43/Val-44, is also hydrolyzed. Sequence analysis of V-8-treated EF-Tu revealed that the N-terminal sequence always starts with Phe-5 (Table I). The amino acid Glu-4 is therefore highly accessible to protease cleavage. On the basis of the molecular weight of the small fragment in the V-8-treated EF-Tu complex, we conclude that peptide 5–64 becomes additionally shortened by about 20 amino acids at its C-terminus. This is probably due to cleavage at position Glu-43. A prolonged incubation time of nucleotide-free EF-Tu with the V-8 protease leads to the complete degradation of the G-domain.

V-8 treatment of nucleotide-free EF-Tu at 4 °C (Figure 3) demonstrates that the decrease of the GDP binding activity in EF-Tu is concomitant with the appearance of the fragment generated by cleavage of Glu-208 ($EF\text{-}Tu_{E208}$). Even upon

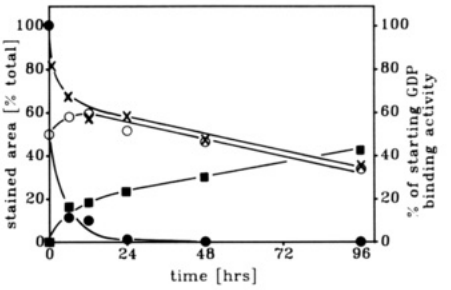

FIGURE 3: Correlation of the GDP binding activity with V-8 cleavage of EF-Tu. Nucleotide-free EF-Tu was incubated with V-8 protease (ratio 1/5) at 4 °C. Ten microliters of the mixture was removed at various times and analyzed by 15% SDS–PAGE and densitometry as described. Intact EF-Tu (●), $EF\text{-}Tu_{N64}$ (○), fragment 209–405 [$EF\text{-}Tu_{E208}$ (■)], and GDP binding activity (×).

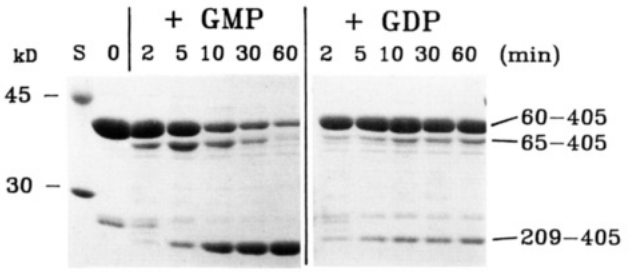

FIGURE 4: Effect of GDP on the cleavage of $EF\text{-}Tu_{R59}$ by *S. aureus* V-8 protease. EF-Tu was pretreated with trypsin (200/1 ratio) for 1 h at 37 °C. The hydrolysis was stopped by the addition of soybean trypsin inhibitor (inhibitor/trypsin ratio 1/5). The resulting $EF\text{-}Tu_{R59}$ was then incubated with V-8 protease (enzyme-substrate ratio 1/5) at 37 °C in the presence of GMP (lanes 2–7) or GDP (lanes 8–12). At the times indicated, 10-μL aliquots were withdrawn and analyzed by 15% SDS–PAGE. Lane S, standard proteins. Only the middle parts of the gels are shown.

excision of the region approximately from residue 44 through Asn-64, the protein remains active in GDP binding. Asparagine-64 is located within the antiparallel β-strand b in the structure of EF-Tu (Jurnak, 1985). We were interested if the conformational change induced by GDP binding leading to the protection of Asn-64 against V-8 treatment (Figure 1) can be observed when the loop region 45–60 is disconnected from this β-strand. Figure 4 demonstrates that in EF-Tu cleaved at Arg-59 Asn-64 is still protected against V-8 protease in the presence of GDP. After 5 min, the amount of fragment deriving from cleavage at Asn-64 is much higher in the presence of GMP than in the presence of GDP whereas the cleavage rate at position Glu-208 is comparable in both cases (Figure 4, lanes 4 and 9).

The accessibility of the most sensitive site in EF-Tu, namely, Arg-59, does not change dramatically upon binding of GDP to nucleotide-free EF-Tu (Figure 1A). The rate of hydrolysis at that position is nearly the same in both forms. This enabled us to follow the hydrolysis at the second target site for trypsin, Lys-275 (Table I), which is located in domain II, in nucleotide-free EF-Tu and EF-Tu·GDP. Figure 5 indicates that the binding of GDP significantly changes the structure around this position in domain II. The rate of hydrolysis at Lys-275 is twice as high in the absence of GDP.

Protease Cleavage Rates for EF-Tu·GDP and EF-Tu·GTP. The replacement of GDP by GTP is expected to cause structural changes in EF-Tu, especially at the binding sites for aa-tRNA and ribosomes. Figure 6 illustrates that the accessibility to protease cleavage of residues located within the region of amino acids 45–60 does not change dramatically upon exchange of GDP for GTP. The only difference could be seen in the hydrolysis rate at Lys-52. The Lys-52/Ala-53

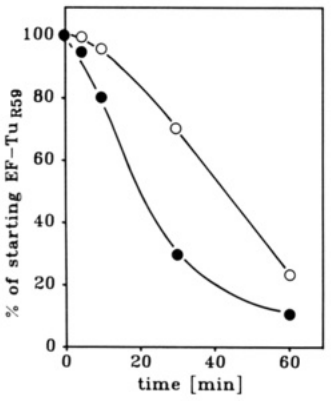

FIGURE 5: Time course of the hydrolysis of EF-Tu_{R59} at Lys-275. Nucleotide-free EF-Tu was digested with trypsin (1/100 ratio) in the presence of GMP (●) or GDP (○). The reaction was performed and evaluated as described under Materials and Methods.

peptide bond was cleaved slightly faster by the Lys-C protease in the GTP form than in the GDP form of the protein. This effect, however, could be due to incomplete binding of GTP under the chosen conditions. As we have shown above, the hydrolysis rate at this position is very dependent on the complete binding of the nucleotide in the GDP/GTP binding site. It is interesting to note that in the presence of GDP the Lys-C protease alternatively cleaves intact EF-Tu at Lys-275, leading to a fragment spanning residues 1–275 (Figure 6B, lanes 2–4). An initial cleavage event at this position could never be observed during the treatment of EF-Tu·GDP with trypsin or of nucleotide-free EF-Tu with the Lys-C protease. This underlines the strong GDP-dependent protection of Lys-52 against hydrolysis.

Influence of the Cleavage of EF-Tu on the Intrinsic and Ribosome-Induced GTPase Activity. To elucidate the function of the effector region in *T. thermophilus* EF-Tu, we tested the GTPase reaction using the different protease-treated proteins. The rate of hydrolysis of EF-Tu-bound GTP can be enhanced by addition of 70S ribosomes (Wolf et al., 1974). To exclude the possibility that differences in the GDP dissociation rates for protease-cleaved and unmodified EF-Tu were rate-limiting during GTP hydrolysis, we followed only one cycle of hydrolysis using an EF-Tu·GTP complex. Under applied conditions, the hydrolysis of GTP by native EF-Tu had a half-time of 23 min (Figure 7A). This could be reduced to 14 min in the presence of an equimolar amount of *E. coli* ribosomes (Figure 7A). In the case of EF-Tu_{R59}, the rate of GTPase was slightly reduced (Figure 7B). Cleavage at Lys-52 had a stronger negative effect on the GTPase activity (Figure 7C). When EF-Tu_{R59} was further cleaved at Lys-275, the GDP binding activity was reduced to 37% of the normal level, and the GTPase activity was nearly abolished (Figure 7D). In all proteolytically cleaved EF-Tu's, the GTPase reaction center could not be significantly activated by the addition of ribosomes. SDS–PAGE analysis of EF-Tu_{K275} revealed that polypeptide 1–59 was shortened at its C-terminus by about seven amino acids probably involving hydrolysis of the Lys-52/Ala-53 peptide bond (Figure 7F, lane 3). We examined the GDP binding and GTPase activities of EF-Tu in which the region 53–59 was excised by combined action of the Lys-C protease and trypsin without affecting position Lys-275 (not shown). The results indicate that the reduced GDP binding activity of EF-Tu_{K275} is due to cleavage at Lys-275 while the strongly reduced GTPase activity is due to the lack of region 53–59. We also investigated the behavior of EF-Tu_{N64} under the same conditions. Whereas the species EF-Tu_{R59} and EF-Tu_{K52} only contain a nick in their polypeptide chain, this

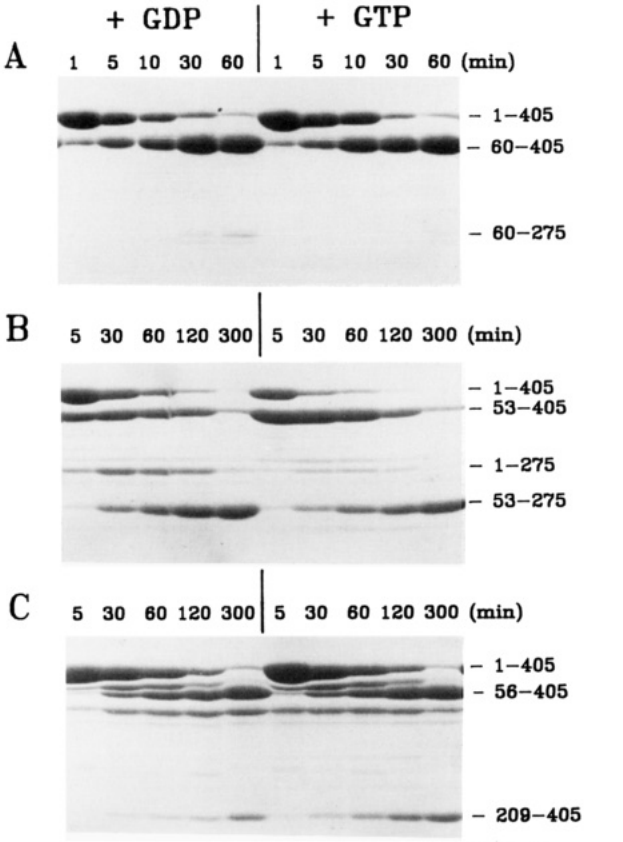

FIGURE 6: Influence of bound GDP or GTP on the initial cleavage of EF-Tu by different endoproteases. Nucleotide-free EF-Tu in the presence GDP (lanes 1–5) or GTP (lanes 6–10) was treated with trypsin (A), Lys-C protease (B), and V-8 protease (C) as describedd in the legend to Figure 1. At the indicated times, aliquots of 10 μL of the mixture were removed and analyzed by 15% SDS–PAGE. Only the middle parts of the gels are shown.

protein lacks a strech of about 20 amino acid residues in the 45–65 region. Despite its full nucleotide binding activity, no GTPase activity could be detected in EF-Tu_{N64}. The addition of ribosomes had no effect on the GTPase activity (Figure 7E).

Affinity Labeling of Trypsin-Cleaved EF-Tu with GDP_{oxi} and GTP_{oxi}. Labeling of native EF-Tu with GTP_{oxi} resulted in a specific modification of Lys-137, Lys-52, and Lys-325, whereas GDP_{oxi} labeled only Lys-52 and Lys-325 (Peter et al., 1988). Figure 8B, lanes 1–3, confirms this expected labeling pattern derived from labeling of native EF-Tu with [U-^{14}C]GDP_{oxi}. By introducing a specific trypsin-induced cleavage at Arg-59, we expected to observe a change in the reactivity of Lys-52 toward modification with both GTP_{oxi} and GDP_{oxi}. The limited tryptic digestion of *T. thermophilus* EF-Tu produces polypeptides 60–405 and 1–59. The large fragment is further degraded by a slower reaction leading to fragments 60–275 and 276–405. Affinity labeling with GDP_{oxi} of the trypsin-treated EF-Tu shows that most of the radioactivity is contained within fragment 1–59 (Figure 8B, lanes 2 and 3) resulting from a modification of Lys-52. Minor reaction sites are found in fragments 60–275 and 276–405, corresponding to modifications of Lys-137 and Lys-325, respectively (lane 3). Labeling of EF-Tu_{R59} with GDP_{oxi} reveals a different distribution of radioactivity (Figure 8B, lanes 4–6). Although fragment 1–59 in the stained gel is clearly visible (Figure 8A, lane 4), the intensity of the corresponding radioactive band is significantly lower than in the case of the intact protein (Figure 8B, lane 2). The labeled EF-Tu_{R59} shows a reactive site in fragment 60–275. This means that upon cleavage at Arg-59, Lys-52 loses its reactivity and a new reactive site is

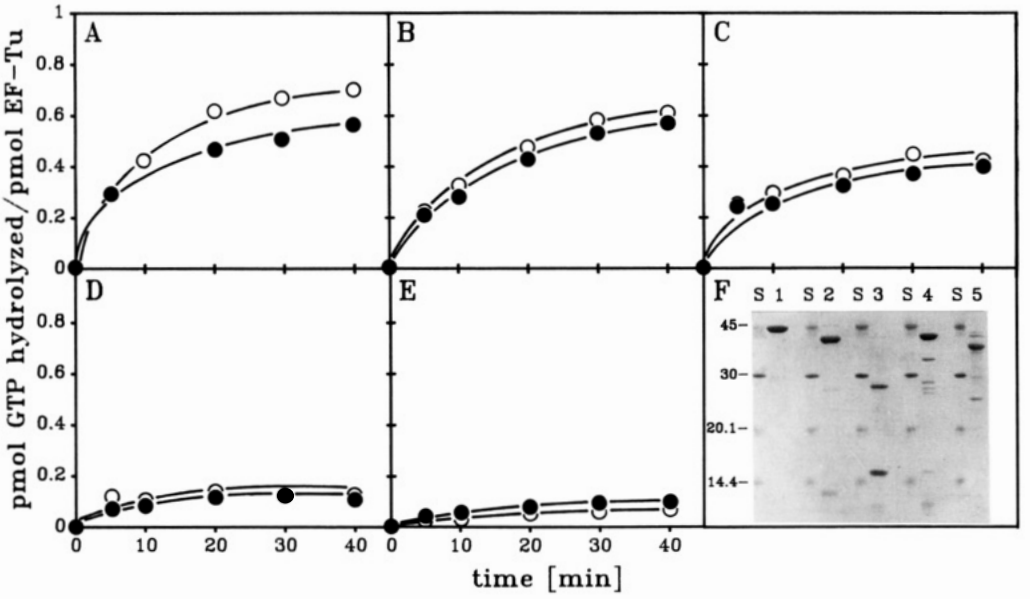

FIGURE 7: GTPase activity of EF-Tu treated with different endoproteases. 10 μM intact EF-Tu was tested for GTPase activity (A) or first pretreated with trypsin in an enzyme/EF-Tu ratio of 1/200 (B) or 1/40 (D), with Lys-C protease (1/4 ratio) (C) for 1 h at 37 °C, or with V-8 protease (1/5 ratio) (E) for 24 h at 4 °C. Reaction mixtures contained 10 μM [γ-^{32}P]GTP (8500 cpm·pmol^{-1}) in the absence (●) or presence (○) of 10 μM *E. coli* 70S ribosomes. The plotted values were corrected by the blank activity determined in the absence of EF-Tu (~5%) and in the absence of EF-Tu and ribosomes (<1%). (F) SDS–PAGE analysis of 10 μg of the starting proteins used in the reactions shown in panels A (lane 1), B (lane 2), D (lane 3), C (lane 4), and E (lane 5). Lane S, standard proteins. The GDP binding activities of the modified proteins were 97% (B), 101% (C), 37% (D), and 80% (E) of the native protein tested in (A).

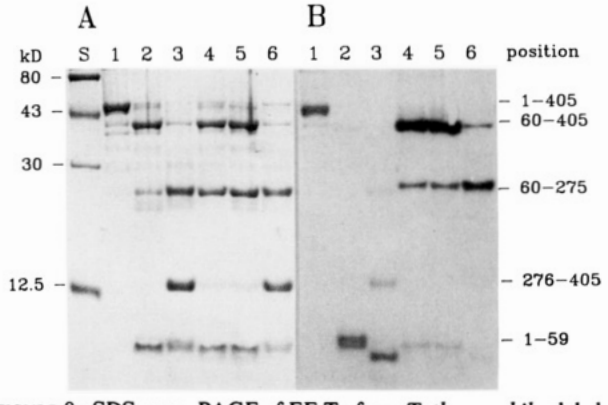

FIGURE 8: SDS–urea–PAGE of EF-Tu from *T. thermophilus* labeled with [U-^{14}C]GDP$_{oxi}$. Coomassie blue staining (A) and autoradiography (B) of the gels are shown. In lanes 1–3, 20 μg of intact EF-Tu was applied; in lanes 4–6, the protein was treated with trypsin prior to labeling (EF-Tu$_{R59}$). After being labeled, the protein was analyzed directly (lanes 1, 4) or treated with 1 μg of trypsin for 30 s (lane 2, 5) or 30 min (lane 3, 6) at 37 °C.

created. Sequence and amino acid composition analysis of the radioactive labeled peptide revealed the N-terminal α-NH$_2$ group of Gly-60 as the site of label attachment. We conclude that the cleavage of EF-Tu at Arg-59 creates a new N-terminus at Gly-60 which is reactive in the cross-linking reaction with both GDP$_{oxi}$ and GTP$_{oxi}$.

Different reactivity of lysine-137 toward GDP$_{oxi}$ and GTP$_{oxi}$ has been observed (Peter et al., 1988). This conformational diversity could not be detected with EF-Tu nicked at Arg-59 (data not shown). Cleavage of the Arg-59/Gly-60 peptide bond has also a pronounced influence on the reactivity of Lys-325 toward oxidized guanosine nucleotides (Figure 8B). Whereas this minor reaction site which is located in the tryptic fragment 276–405 can be clearly detected with native EF-Tu (lane 3), it is absent in the case of EF-Tu$_{R59}$ (lane 6).

Mapping the Effector Loop by Affinity Labeling. Four enzymes were used to cleave EF-Tu specifically within the region of residues 40–75. The positions of the cleavage sites are summarized in Figure 9. The N-termini Asp-44, Ala-53, Glu-56, Gly-60, Thr-65, Thr-72, and Arg-75 could be produced. EF-Tu species specifically cleaved in that way were subjected to affinity labeling with GDP$_{oxi}$. The reaction products were analyzed by limited trypsin cleavage, polyacrylamide gel electrophoresis, and autoradiography (Figure 10). In all cases, a prominent new reaction site is introduced into the 50–60 region of EF-Tu by proteolytic cleavage. Yield of the affinity label depends on the position of the new N-terminal amino acid in the loop sequence (Figure 9). The N-terminus at Glu-56 is most reactive, placing this residue closest to the bound nucleotide, followed by intermediate reactivity of the Gly-60 and Ala-53 N-termini. Not reactive are the N-terminal amino acids Ala-1, Phe-5, Thr-65, Thr-72, Arg-75, Tyr-209, and Thr-276. Another enzyme which was found to cleave EF-Tu exclusively in the region spanning residues 45–70 was chymotrypsin (not shown). Treatment of the resulting labeled chymotryptic fragments with trypsin revealed again that the N-terminal amino acid carries the radioactivity, as shown for labeled EF-Tu$_{E55}$ and EF-Tu$_{K52}$. Because of some unspecific cleavage events in the respective region, we were not able to determine the exact cleavage sites.

Discussion

Affinity Labeling of EF-Tu Cleaved at Arg-59. All known eukaryotic and prokaryotic GTP binding elongation factors contain a conserved arginine/glycine peptide bond which is sensitive to trypsin hydrolysis (Möller et al., 1987). The possibility of cleaving *T. thermophilus* EF-Tu at Arg-59 enabled us to perform the affinity labeling with GDP$_{oxi}$ or GTP$_{oxi}$ with a nicked protein. The ability of Lys-52 to react toward the bound nucleotide was lost completely upon cleavage at Arg-59 due to dislocation of the region 45–59. This result is compelling evidence that the cross-linking event with Lys-52 requires a defined location of the loop region 45–60 with respect to the bound nucleotide.

In the reaction of GDP$_{oxi}$ with EF-Tu$_{R59}$, we found only one modified NH$_2$ group, namely, the N-terminus of the Gly-60–Glu-405 fragment. The cross-linking yield at this position

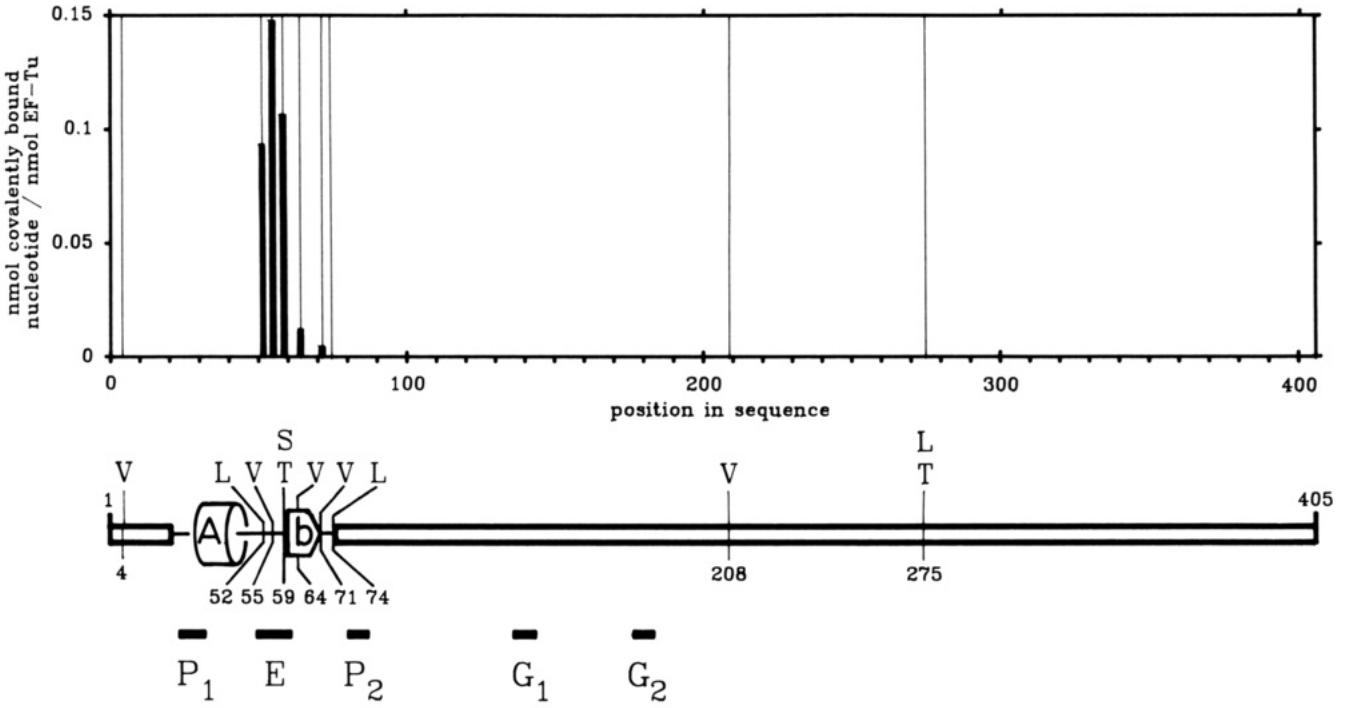

FIGURE 9: Schematic presentation of initial cleavage sites for different endoproteases along the *T. thermophilus* sequence and reactivities of the new N-termini during affinity labeling with GDP_{oxi}. T, trypsin; V, *S. aureus* protease V-8; L, Lys-C protease; S, submaxillary protease cleavage sites. Reactivities were determined by densitometry of the Coomassie blue stained protein and the respective autoradiograms. The secondary structure elements around the effector region (E) are indicated. The cylinder corresponds to the α-helix A and the arrow to the antipararllel β-strand b in the EF-Tu structure (Jurnak, 1985). P_1 and P_2 are the phosphoryl binding loops 1 and 2, G_1 and G_2 are the guanine binding regions 1 and 2.

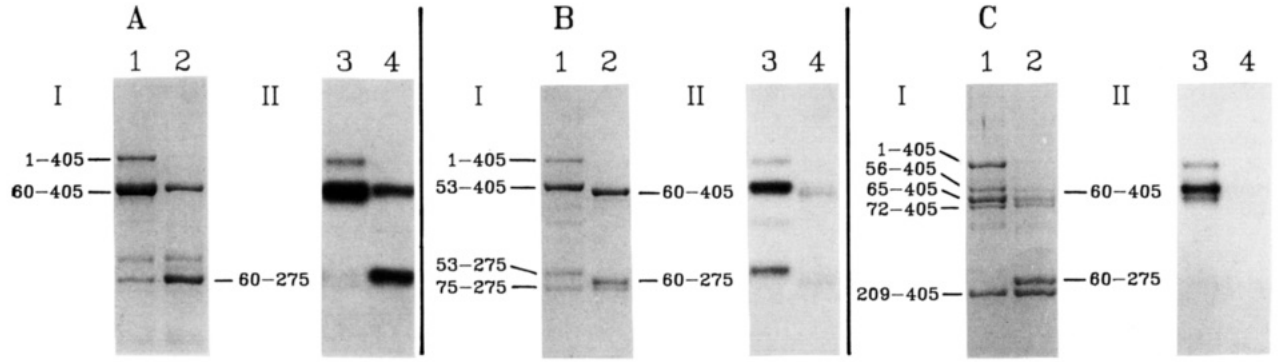

FIGURE 10: SDS–urea–PAGE analysis of proteolytically cleaved and [β-^{32}P]GDP_{oxi}-labeled EF-Tu. Twenty micrograms of EF-Tu·[β-^{32}P]GDP was treated with submaxillary protease (A), Lys-C protease (B), or *S. aureus* protease V-8 (C), respectively, and subjected to affinity labeling. After treatment with 1 μg of trypsin for 30 min (lane 2 in A) or for 30 s (lanes 2 in panels B and C), the reaction products were analyzed by 12.5% SDS–urea–PAGE. Positions of fragments derived from proteolytic cleaved and affinity- labeled EF-Tu (I) and positions of altered fragments upon an additional cleavage with trypsin (II) are given. Lanes 3 and 4 show the autoradiograms of the respective lanes 1 and 2 of the stained gel. Only the upper part of the gel is presented.

was 4–5-fold higher than that at Lys-52 during labeling of the native protein under identical conditions. This effect, however, could be due to different p*K* values of the alternatively reactive α- and ϵ-amino groups and does not necessarily reflect the better accessibility of the oxidized ribose to the N-terminal glycine residue.

The residue lysine-325 located in C-terminal domain III is modified to a low extent by both GDP_{oxi} and GTP_{oxi} in native EF-Tu (Peter et al., 1988). In the same experiment with the nicked protein, it was not reactive toward oxidized guanine nucleotides. We conclude that the complete lack of Lys-325 modification by GDP_{oxi} or GTP_{oxi} in $EF\text{-}Tu_{R59}$ reflects an altered location of this residue with respect to the nucleotide binding site.

Limited Protease Treatment of EF-Tu. We have found that the sensitivity to endoproteases of certain residues in the region of the protein spanning amino acids 40–65 depends much more on whether any nucleotide is bound to the protein than on the nature of the bound nucleotide, i.e., GDP or GTP. Lys-52 is only sensitive to proteolytic cleavage when EF-Tu is in a nucleotide-free form. This, together with the results of affinity labeling of native EF-Tu, shows that the side chain of Lys-52 is near the ribose moiety when EF-Tu is bound by GDP. This interaction is not present in nucleotide-free EF-Tu.

Asparagine-64 in β-strand b of the EF-Tu structure was completely protected against V-8 protease cleavage when GDP or GTP were bound to the protein. This effect remains unchanged even after cleaving EF-Tu at Arg-59, indicating an interaction of β-strands b and c as delineated in the model of Jurnak (1985). This geometry of the antiparallel β-strands b and c is in agreement with the structure of $p21^{ras}$ (de Vos et al., 1988) and enabled us also to explain the cross-linking event of Lys-52 with the bound oxidized nucleotide in the intact protein.

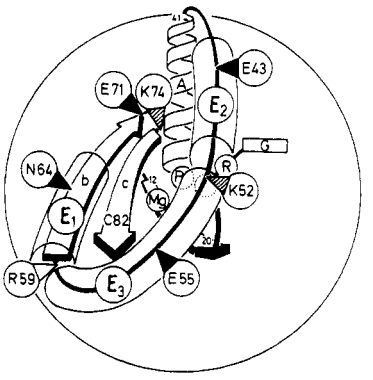

FIGURE 11: Proposed model of the effector interacting region in *T. thermophilus* EF-Tu. The location of the proteolytic cleavage sites around the effector region within the first 83 amino acid residues of the EF-Tu G-domain is shown. The α-helix A and the neighboring β-strands b and c are labeled. Solid arrows indicate V-8 cleavage sites, the hatched arrows indicate the Lys-C protease cleavage sites, and the open arrow points to the trypsin and submaxillary protease cleavage site. E_1, putative aa-tRNA interaction site; E_2, putative ribosome interaction site; E_3, GTPase activating region; E, glutamic acid; K, lysine; R, arginine; N, asparagine; C, cysteine.

Cleavage of the EF-Tu G-domain at positions other than those indicated in Figure 11 leads to complete degradation of its structure, indicating that the antiparallel β-strands b and c are important for the stabilization of the entire G-domain.

Apart from the strong influence of the binding of GDP on the conformation of certain parts of the G-domain, additional long-range effects originate from the bound nucleotide. The position Glu-208 can be hydrolyzed much more efficiently in the nucleotide-free form of EF-Tu than in the EF-Tu·GDP complex. Binding of GDP probably induces a conformational change around the hinge region between domains I and II. Additionally, the peptide bond at Lys-275 in domain II is cleaved more efficiently by trypsin in the apoprotein than in EF-Tu·GDP.

The aa-tRNA Interaction Region (E_1). We have previously shown that, upon introducing a nick at position Arg-59, *T. thermophilus* EF-Tu does not participate in poly(U)-directed poly(Phe) synthesis but still remains active in ternary complex formation (Gulewicz et al., 1981). Recent results show that *E. coli* EF-Tu·GTP even after removing the peptide Ala-45–Arg-58 still forms a ternary complex with aa-tRNA with only a slightly reduced binding affinity (unpublished results). The region corresponding to amino acids 45–59 in *T. thermophilus* EF-Tu therefore cannot be essential for aa-tRNA binding.

We propose that the interaction site for aa-tRNA on the G-domain of EF-Tu is located within the β-strand b spanning residues 60–70. It is susceptible to protease treatment, compelling evidence that this region is a candidate for interaction with a macromolecule, and an α-NH_2 group created in this region cannot be cross-linked to the bound nucleotide, showing the distance to the GDP binding site. Additionally, in $p21^{ras}$, the effector binding site was found to be located in the respective β-strand b rather than in the loop region 20–31 (Sigal et al., 1986; de Vos et al., 1988) which corresponds to residues 40–60 in EF-Tu.

Biochemical evidence focuses on His-66 located within β-strand b being involved in interaction with aa-tRNA (Duffy et al., 1981; Jonák et al., 1984; Metz-Boutigue et al., 1989). Modification of Cys-81 in the adjacent β-strand c of *E. coli* EF-Tu (Jonák et al., 1982) as well as the corresponding residues in the EF-Tu's from *T. thermophilus* (Peter et al., 1989) and *Bacillus stearothermophilus* (Jonák et al., 1986) blocks aa-tRNA binding. The region around the corresponding residue in G-proteins was postulated to be involved in binding the γ-phosphate group of GTP (la Cour et al., 1985) and was called the S-box (switch-box), indicating that a dramatic conformational change occurs here caused by the exchange of GDP versus GTP (Holbrook & Kim, 1989), which is one of the prerequisites for a putative effector interacting region.

The Ribosome Interaction Region (E_2). It is known from studies with the isolated G-domain of *E. coli* EF-Tu that the GTPase center can be activated by the addition of 70S ribosomes as observed with the native protein (Parmeggiani et al., 1987). Therefore, the G-domain must contain a ribosome interaction site. We found that in the case of native *T. thermophilus* EF-Tu the addition of 70S ribosomes also leads to an increased GTP hydrolysis. When a part of the stretch of amino acids 44–64 is removed, the intrinsic GTPase activity is destroyed and cannot be reactivated by addition of ribosomes. This result indicates that this region is involved in the activation of the GTPase center as well as in ribosome binding. This was also found for eEF-Tu from rabbit reticulocytes where Slobin et al. (1981) could not detect a ribosome-dependent activity upon treating the protein with trypsin.

Another elongation factor which requires ribosome binding for the activation of its GTPase center is elongation factor G (EF-G). Nilsson and Nygård (1988) showed that limited tryptic cleavage of the "eukaryotic EF-G" (EF-2) at the hydrolysis-sensitive arginine residue resulted in a loss of high-affinity ribosome binding.

Ryazanov et al. (1988) reported that the phosphorylation of EF-2 blocks the translocation of the peptidyl-tRNA on the ribosome on the ribosome by interrupting the interaction of EF-2 with the ribosome and therefore the signal for GTPase activation (Ryazanov & Davidova, 1989). The phosphorylation sites, Thr-53, Thr-56, and Thr-58, are located in the region which corresponds to residues 47–52 in *T. thermophilus* EF-Tu. The regions in the two proteins display some homology (Kohno et al., 1986) and could play similar roles.

The GTPase Activator Region (E_3). EF-Tu displays an intrinsic GTPase activity. The EF-Tu GTPase center can be activated by addition of aa-tRNA and still further increased by adding ribosomes (Wolf et al., 1974). This additive effect indicates that both macromolecules interact with different sites on EF-Tu. By testing the GTPase activity of various EF-Tu's cleaved at different sites in the effector region, we found that the hydrolysis rate is only slightly reduced by introducing a nick into the polypeptide chain. Jacobson and Rosenbusch (1977) have made a similar observation for trypsin-treated *E. coli* EF-Tu. Treatment of nucleotide-free EF-Tu with the *S. aureus* V-8 protease leads to the removal of about 20 amino acid residues (44–64). Although this protein is still fully active in nucleotide binding, the ability to hydrolyze GTP is lost completely.

In light of these results, the region 52–59 functions as a transducer for the signal "GTPase activation" rather than as an effector binding site. It has been shown for *E. coli* EF-Tu that methylation of Lys-56 located in the corresponding *E. coli* sequence and thought to influence the interaction with aa-tRNA (Laursen et al., 1981) mediates the aa-tRNA-induced GTPase activity of EF-Tu without affecting the binding of aa-tRNA to EF-Tu·GTP (van Noort et al., 1986). Additionally, our finding that an α-amino group created within this region can be cross-linked to the bound GDP_{oxi} underlines its vicinity to the nucleotide binding site.

It is conceivable that the interaction of aa-tRNA with EF-Tu changes the conformation of the loop region 52–59, leading to an altered accessibility of the basic residues toward

trypsin since these residues are protected against cleavage in the procaryotic (Arai et al., 1976; Pingoud et al., 1982) and eucaryotic EF-Tu's (Slobin et al., 1983; Miyazaki et al., 1988) upon binding of aa-tRNA.

In general, the interaction of a GTP binding protein with its effector molecule would result in a conformational change of the E_3 region (Figure 11), i.e., the region preceding the antiparallel β-strand b present in all GTP binding proteins. The conformational change could lead to an altered interaction of the loop region with either the nucleotide or the adjacent phosphate binding loop (the G-loop) which was shown to play an essential role for GTPase activity in *E. coli* EF-Tu (Jaquet & Parmeggiani, 1988), the YPT protein (Wagner et al., 1987), and the ras proteins (Barbacid, 1987).

Structural and Functional Implications for the Cycle of GTP Binding Proteins. The model presented in Figure 11 summarizes our present view of the function of the region spanning residues 40–70. The functional cycle of EF-Tu can be described by two structural changes. The first conformational transition occurs when GTP replaces GDP as a consequence of the activation by EF-Ts. Only in this structure can EF-Tu interact with its effector molecule, aa-tRNA. The region 60–70 (E_1) fulfills the requirements for a putative aa-tRNA interaction site. On the basis of analogy with EF-2, the region 40–50 (E_2) in EF-Tu could be involved in the interaction with the ribosome which acts as a co-effector in the functional cycle.

The second conformational change only occurs in the EF-Tu·GTP form and is triggered by the binding of effector and/or co-effector. As a result, the region 51–59 (E_3) undergoes a dramatic structural change leading to the activation of the GTPase center. All known GTP binding proteins follow the same functional cycle (Allende, 1988). The analogy of a molecular switch for signal tranduction has been employed for all procaryotic and eukaryotic GTP binding proteins. All proposed functional models, which are based on the crystal structure of the *E. coli* EF-Tu·GDP G-domain (Jurnak, 1985; la Cour et al., 1985), lack what we believe to be one of the most important regions of the protein responsible for the GTPase signal tranduction. We postulate that in all GTP binding proteins having a homologous functional unit, namely, the G-domain, there is a loop preceding the only antiparallel β-strand which interacts with the nucleotide and is involved in the regulation of GTPase activity.

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Registry No. GTP, 86-01-1; GDP, 146-91-8; GTPase, 9059-32-9.

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Phospholipid Monolayers at the Triolein–Saline Interface: Production of Microemulsion Particles and Conversion of Monolayers to Bilayers

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ABSTRACT: Interfacial tensions of phospholipid monolayer at the triolein (TO)–saline interface were measured. The adsorption isotherms and the interfacial pressure–molecular area curves were evaluated on the basis of the measurements. Phosphatidylcholine (PC) forms a highly condensed monolayer, with a large lateral attractive interaction; phosphatidylethanolamine (PE) and phosphatidylserine (PS) form expanded monolayers with smaller lateral interaction energies. At the lowest interfacial tension (the highest interfacial pressure), the mole fractions of PC, PE, and PS in the monolayers are estimated as 0.95, 0.73, and 0.88, respectively. Therefore, PC forms the most stable monolayer at the interface. These results are consistent with the finding that the stable TO particles in aqueous solution were produced by using PC as an emulsifier, and PE and PS did not stabilize the particles. The phase diagram of TO and PC mixtures in saline obtained from theoretical considerations predicts the equilibrium conversion of the monolayers on TO particles to bilayers. This process may be closely related to the transformations of very low density lipoproteins and chylomicrons to high-density lipoproteins in plasma. The particle sizes of the emulsion are calculated theoretically as a function of PC mole fracton in the TO–PC mixture and compared with the experimental values obtained from quasi-elastic light scattering (QLS) measurements.

Lipoproteins may be viewed as emulsion particles whose component lipids are in dynamic equilibrium with lipids in each class of lipoproteins as well as with those in the membranes of various tissues. Both chylomicrons and nascent very low density lipoproteins (VLDLs),[1] formed in the small intestine and the liver cells, respectively, are large (100–1000-nm) emulsion particles composed of triglyceride-rich cores stabilized by surface phospholipid (mainly phosphatidylcholine) monolayers containing a very small amount of several different apoproteins (Patton et al., 1984). In plasma, these particles attach to capillary endothelium, where lipoprotein lipase promotes triglyceride hydrolysis (Mjos et al., 1975; Vigne & Havel, 1981; Redgrave & Small, 1979). The digestion of triglyceride shrinks the core to produce the core remnant particle (VLDL or chylomicron remnants) (Atkinson & Small, 1986; Miller & Small, 1983a): As the shrinkage proceeds, the redundant surface monolayers change into bilayers and finally separate from the particles as surface remnants (Tall & Small, 1980). The surface remnants, mainly composed of phospholipids, fuse with some elements of the high-density lipoprotein (HDL) system (Tall et al., 1982). Thus, the surface monolayers play important roles in the particle stabilization and in the conversion of emulsion particles into bilayers.

Emulsion models for triglyceride-rich lipoproteins, prepared by vortexing egg phosphatidylcholine, cholesterol, and triolein (TO) in excess water, have been characterized: Phosphatidylcholine is found exclusively in the surface monolayers separated from the neutral lipid cores. The surface composition of TO is ca. 3 mol % (Miller & Small, 1982, 1983a,b). The β-carbonyl group of TO is less hydrated than the α groups (Hamilton & Small, 1981). Also, addition of cholesterol increases the size of emulsion particles (Miller & Small, 1983b).

In this connection, we have measured the interfacial tension of phospholipid films at the TO–saline interface to evaluate monolayer compositions, lateral interaction parameters, and phase behaviors of TO–phospholipid mixtures in water by using thermodynamic approaches. On the basis of our results,

[1] Abbreviations: VLDL, low-density lipoprotein; HDL, high-density lipoprotein; TO, triolein; PC, egg yolk phosphatidylcholine; PE, egg yolk phosphatidylethanolamine; PS, bovine brain phosphatidylserine; QLS, quasi-elastic light scattering.

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