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Properties of a genetically engineered G domain of elongation factor Tu

(protein biosynthesis/guanine nucleotide-binding protein/GTPase/protein p21/site-directed mutagenesis)

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ABSTRACT **The G domain of elongation factor Tu (EF-Tu), representing the N-terminal half of the factor according to its three-dimensional model traced at high resolution, has been isolated by genetic manipulation of *tufA* and purified to homogeneity. The G domain, whose primary structure shares homology with the eukaryotic protein p21, is capable of supporting the basic activities of the intact molecule (guanine nucleotide binding in 1:1 molar ratio and GTPase activity). However, it is no longer exposed to the allosteric mechanisms regulating EF-Tu. The G-domain complexes with GTP and GDP display similar K_d' values in the μM range, in contrast to EF-Tu that binds GDP much more tightly than GTP. Its GTPase shows the characteristics of a slow turnover reaction (0.1 mmol·sec^{-1}·mol^{-1} of G domain), whose rate closely corresponds to the initial hydrolysis rate of EF-Tu·GTP in the absence of effectors and lies in the typical range of GTPase of the p21 protein. Of the EF-Tu ligands only the ribosome displays a clear effect enhancing the G-domain GTPase. Our results suggest that the middle and C-terminal domain play an essential role in regulating the activity of the N-terminal domain of the intact molecule as well as in the interactions of EF-Tu with aminoacylated tRNA, elongation factor Ts, and kirromycin. With the isolation of the G domain of EF-Tu, a model protein has been constructed for studying and comparing common characteristics of the guanine nucleotide-binding proteins.**

The guanine nucleotide-binding proteins have attracted much attention, particularly since a number of proteins regulating different cell functions (proliferation, hormone response, neurotransmission, and protein biosynthesis) were found to belong to this family of proteins (for example, see refs. 1–3). Elongation factor Tu (EF-Tu) from *Escherichia coli* is one of the best characterized guanine nucleotide-binding proteins (4, 5) and the only one whose three-dimensional structure has been partially elucidated at high resolution (6, 7). Its 393-amino acid polypeptide chain is folded in three distinct domains. The N-terminal domain (residues 1–200) binding GDP or GTP has primary structure features common to the ras protein p21 and other nucleotide-binding enzymes (6–11). Of the three domains of EF-Tu, the N domain displays an α/β type structure, traced at high resolution (0.27–0.29 nm), consisting of six α-helices alternating with six β-strands, typical for the class of nucleotide-binding proteins (6, 7). Fig. 1 is a three-dimensional cartoon derived from x-ray diffraction patterns of mildly trypsinized EF-Tu·GDP crystals (modified from ref. 7).

Our present knowledge of the function, structure, and genetics of *E. coli* EF-Tu makes it a suitable model for the investigation of function–structure relationships (4, 5). Its activity in the elongation cycle of protein synthesis, as the carrier of aminoacylated (aa) tRNA to ribosomes, is regulated by a complex sequence of allosteric mechanisms. As with other guanine nucleotide-binding proteins, the active conformation of EF-Tu, which can form a complex with aa-tRNA, occurs only in the presence of GTP. Binding of this ternary complex to the ribosomal aminoacyl site is associated with GTP hydrolysis catalyzed by EF-Tu (12). GTP hydrolysis induces a different EF-Tu conformation with low affinity for aa-tRNA and ribosomes, leading to the release of the EF-Tu·GDP complex from the ribosome and subsequent peptide bond formation between aa-tRNA in the acceptor site and peptidyl-tRNA in the peptidyl site. Besides its function in protein synthesis, EF-Tu forms one of the subunits of the replicase of an RNA virus (13). EF-Tu has been shown to interact with the adenylate cyclase (14), resembling the GTP-binding proteins involved in hormone response in vertebrates (2) and the ras products in *Saccharomyces cerevisiae* (15). To facilitate the comparison of the properties of different nucleotide-binding proteins, we have constructed a gene encoding the EF-Tu N-terminal domain, whose product has been designated the G domain, by deleting part of *tufA* via oligonucleotide-directed mutagenesis.

MATERIALS AND METHODS

The *E. coli* strain used throughout this report was the 71/18 (Δ{*lac-pro*}F′*lac*I^q*lac*Z ΔM15 *pro*$^+$ *supE*). Mutagenesis was carried out on *tufA* inserted in the pEMBL9$^+$ (16), and overexpression of the mutated *tufA* was in the "runaway" vector pCP40 (17). The cells, grown in rich medium containing ampicillin and kanamycin, were centrifuged and suspended (1 g/3 ml) in 50 mM Tris·HCl, pH 7.6/700 mM KCl/5 mM $MgCl_2$/15 mM 2-mercaptoethanol/1 mM phenylmethanesulfonyl fluoride/15% (vol/vol) glycerol and sonicated. The cell extract was centrifuged for 3 hr at 100,000 × *g* at 2°C, and the supernatant (S-100) was chromatographed on DEAE-Sepharose (Pharmacia) using a linear 60–220 mM KCl gradient in 50 mM Tris·HCl, pH 7.6/5 mM $MgCl_2$/7 mM 2-mercaptoethanol/15% (vol/vol) glycerol. The active fractions were concentrated and chromatographed on Ultrogel AcA44 (IBF) in 50 mM Tris·HCl, pH 7.6/5 mM $MgCl_2$/150 mM KCl/7 mM 2-mercaptoethanol/15% (vol/vol) glycerol. As the last purification step, fast protein liquid chromatography (FPLC) (Pharmacia) was carried out on a Mono Q column, using a

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Abbreviations: EF-Tu, elongation factor Tu; EF-Ts, elongation factor Ts; aa-tRNA, aminoacylated tRNA; FPLC, fast protein liquid chromatography.

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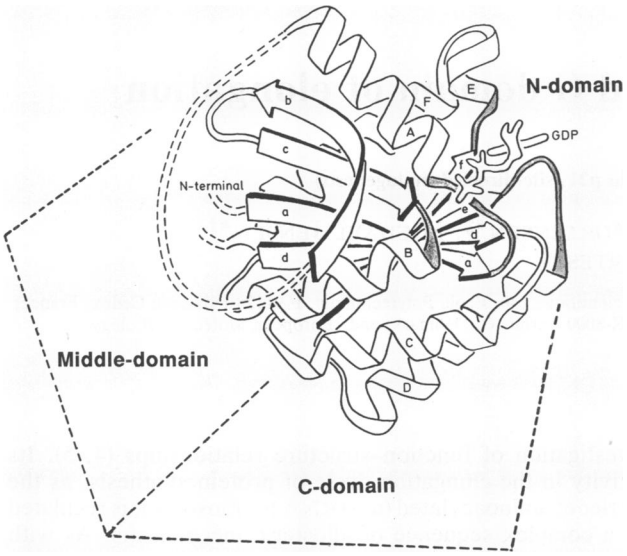

FIG. 1. Three-dimensional model of the N domain in the context of the EF-Tu molecule (modified from ref. 7). Arrows represent β-strands and curled ribbons represent α-helices. Dotted lines schematically indicate the presence of the adjacent middle and C-terminal domains and do not intend to assign the relative positions of these two domains with respect of the N domain.

linear 250–350 mM KCl gradient in 50 mM Tris·HCl, pH 7.6/5 mM $MgCl_2$/7 mM 2-mercaptoethanol.

GDP binding assay on nitrocellulose filters (Sartorius, SM 11306, 0.45 μm) and GTPase activity measured as liberation of P_i were performed as described (18). All biological components, materials, and methods not mentioned in this section were as reported (18).

RESULTS AND DISCUSSION

Strategy for the Construction and Overproduction of the G Domain. To isolate the N-terminal domain of EF-Tu as an independent protein, we have inserted a 2-kilobase DNA fragment containing the structural *tufA* gene in the unique *Sma* I site of pEMBL9$^+$, a vector harboring the origin of replication of phage F1 and thus susceptible to encapsidation and secretion as single-stranded DNA after F1 superinfection (16). In the resulting vector pEMBL9$^+$*tufA*$^{P-}$ ($^{P-}$ indicates that the orientation of *tufA* is opposite to *lacP*), the expression of *tufA* occurs under control of its vicinal promotor positioned in the flanking intergenic region and in the terminal portion of the *fus* gene (19). Deletion of the two other domains (middle domain and C-terminal domain) according to the three-dimensional model has been achieved by looping out their corresponding gene fragment (570 base pairs) with a synthetic 17-mer oligodeoxynucleotide (5′-GGAACCA-G_ΔGCTAATTGC-3′, where $_\Delta$ indicates the deletion site). Its 5′ half hybridized to bases 603–610 of the structural gene, and its 3′ half hybridized to the last two bases (positions 1180 and 1181) of the codon for the C terminus glycine-393, the termination codon, and the first four bases of the flanking region. Therefore, the deletion concerns the polypeptide chain from glutamic acid-203 to leucine-392. Mutagenesis was carried out using the gapped duplex method (20). Selection of the transformed colonies carrying the deleted *tufA* gene was performed by cell colony hybridization. The mutation was confirmed by sequencing the DNA using the dideoxy method.

To overcome the low expression of the constructed vector pEMBL9$^+$*tufA*(Δ610-1180)$^{P-}$, we have cloned *tufA* (Δ610-1180) into the *Eco*RI–*Hin*dIII polylinker site of the runaway expression vector pCP40, in which it is under λP_L promoter control (17). In this system, shift to 42°C induces an overproduction of the G domain, in some cases up to 50% of the total cellular protein of the host *E. coli* strain 71/18. Fig. 2 schematically shows the method adopted for genetically engineering the G domain.

Purification of the G Domain. Fig. 3*A* illustrates the results of $NaDodSO_4$/PAGE of total cell proteins obtained with cracking buffer (37), after overproduction of the G domain. Roughly one-third of the G domain so produced is soluble, whereas the remaining two-thirds are insoluble and precipitate, possibly a consequence of incomplete folding. The insoluble product is removed by centrifugation for 90 min at 100,000 × *g* (data not shown). Fig. 3*B* shows the $NaDodSO_4$/polyacrylamide gel of the G domain purified by chromatography on DEAE-Sepharose, by filtration on Ultrogel AcA44, and by FPLC on Mono Q. The G domain is eluted immediately after EF-Tu and overlaps the elongation factor G peak on DEAE-Sepharose. The filtration step on Ultrogel AcA44 yields a G domain at least 80% pure, free of any contaminating EF-Tu and elongation factor G. After FPLC chromatography the G domain is over 96% pure. The concentrated, active fractions are stable for months when stored in 50 mM Tris·HCl buffer, pH 7.6/5 mM $MgCl_2$/50 mM KCl/7 mM 2-mercaptoethanol/20 μM GDP/50% (vol/vol) glycerol at −35°C.

The G Domain Can Bind GDP and GTP. Guanine nucleotide-binding experiments show that the isolated G domain is able to bind GDP and GTP, confirming the evidence obtained from the x-ray diffraction studies that locate the binding site for GDP in the N domain of EF-Tu (6, 7). Saturation experiments (Fig. 4) show that GDP or GTP binding to the G

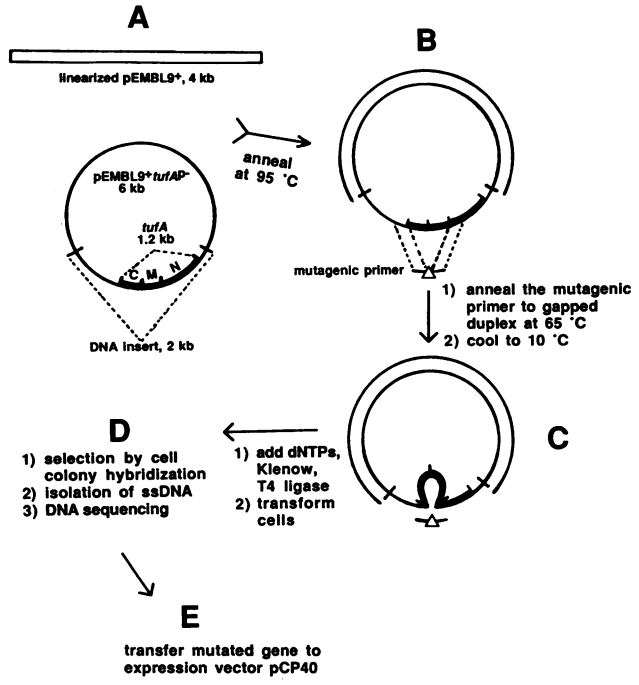

FIG. 2. Schematic representation of the genetic manipulations carried out for the construction of the G domain. (*A*) Linearized pEMBL9$^+$ and single-stranded DNA of pEMBL9$^+$*tufA*$^{P-}$. N, M, and C indicate the *tufA* DNA coding for N, Middle, and C domains of EF-Tu, respectively. (*B*) Gapped duplex with the single-stranded DNA insert containing *tufA* and the mutagenic primer. Dotted lines refer to the two distant sequences flanking the single-stranded DNA to be deleted and complementary to the primer. (*C*) Hybridization between the mutagenic primer and the single-stranded DNA looping out the portion coding for the Middle and C domain of EF-Tu. (*D* and *E*) Synopsis of the steps following mutagenesis. The size of the different components is purely illustrative and does not reflect the real relationship.

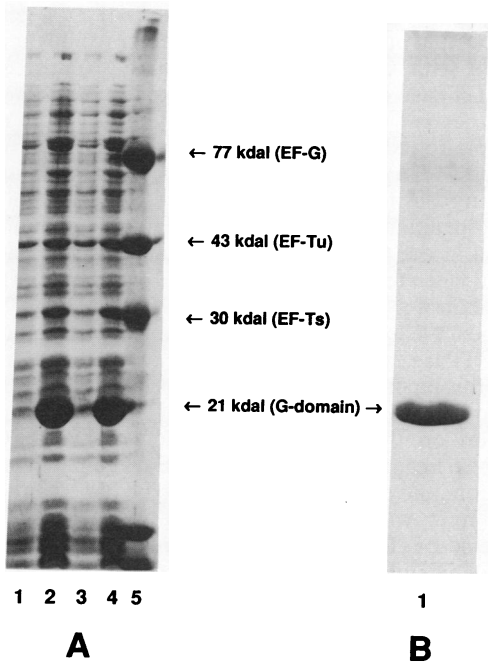

FIG. 3. Overproduction of the G domain in the *E. coli* cell and purity of the isolated G domain. (*A*) NaDodSO$_4$/PAGE of the total cell protein extract of two *E. coli* clones prior (lanes 1 and 3) and after (lanes 2 and 4) induction of λP_L and runaway replication. Lane 5, molecular size markers. (*B*) NaDodSO$_4$/PAGE of the G domain after purification by DEAE-Sepharose chromatography, by Ultrogel AcA44 filtration, and by FPLC chromatography on MonoQ. Kdal, kDa.

domain corresponds to a stoichiometry approaching the 1:1 molar ratio found with EF-Tu. The dissociation constants (K_d') of the G-domain complexes with either GTP or GDP are similar (Table 1). This represents a radical change of the situation found with EF-Tu. In fact, in the intact molecule the main feature of the nucleotide binding is the difference between the properties of the two complexes. Their K_d' values differ by two orders of magnitude (Table 1). Thus, compared with EF-Tu, it is the K_d' of the G domain·GDP complex that shows the more profound change, displaying values higher than those of EF-Tu·GDP by two orders of magnitude, whereas the K_d' of the G domain·GTP complex is only a few

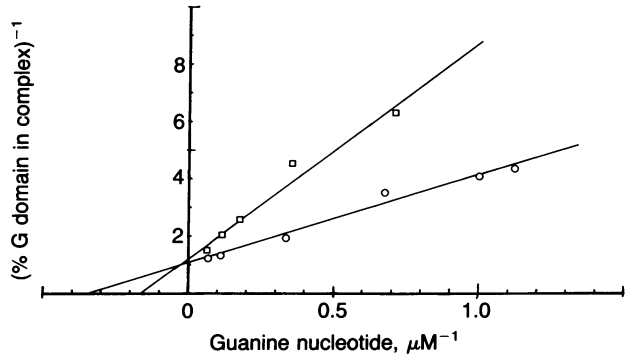

FIG. 4. Inverse plot of GDP or GTP binding to G domain as a function of the guanine nucleotide concentration. Reaction mixtures contain, in a final volume of 28 μl of standard buffer, 2 μM nucleotide-free G domain, [^{3}H]GDP (specific activity, 600 cpm·pmol^{-1}) (○) or [γ-^{32}P]GTP (specific activity, 1385 cpm·pmol^{-1}) (□) at the indicated concentrations. After incubation at 30°C for 10 min, 20-μl samples were withdrawn, and binding of the guanine nucleotide to the G domain was determined by filtration on nitrocellulose filters as described in Table 1.

Table 1. Comparison of the apparent dissociation constants (K_d') of the GTP and GDP complexes of the G domain relative to EF-Tu and p21

Protein complex	K_d', μM	
	0°C	30°C
G domain·GTP	8	4
G domain·GDP	2	3
EF-Tu·GTP	0.3–0.8	0.6
EF-Tu·GDP	0.001–0.007	0.006
p21·GTP	0.02–0.05	
p21·GDP	0.02–0.05	

The K_d' values were determined in a reaction mixture containing, in a final volume of 28 μl, 2 μM nucleotide-free G domain in standard buffer (50 mM imidazole acetate, pH 7.6/50 mM NH_4Cl/10 mM $MgCl_2$/1 mM dithiothreitol), [^{3}H]GDP (specific activity, 600 cpm·pmol^{-1}), [γ-^{32}P]GTP (specific activity, 1385 cpm·pmol^{-1}) in a concentration between 0.8 and 17 μM. Complex formation was allowed to take place during a 30-min incubation at 0°C or a 10-min incubation at 30°C. A sample of 20 μl was withdrawn and spotted on nitrocellulose filters. After washing immediately with 3 ml of cold standard buffer, the filters were dried, and the radioactivity was measured in toluene containing 2,5-diphenyloxazole (31) using an Intertechniques Scintillation Spectrometer Model 4000. The K_d' values for the G-domain complexes are the average of several experiments. The range at 0°C for the K_d' of EF-Tu·GDP and EF-Tu·GTP are reported from refs. 21–23, the values at 30°C are from ref. 22, and the range for the K_d' of p21·GDP and p21·GTP from ref. 24. G domain was freed from bound GDP following the procedure applied for EF-Tu·GDP (25).

fold higher than that of EF-Tu·GTP. It is worth mentioning that the K_d' values of the complexes of the Ha-*ras* protein p21 with GTP and GDP are also similar, though they are lower than in the case of the G domain (Table 1).

The higher affinity of EF-Tu toward GDP is accounted for by the locked conformation of EF-Tu·GDP, characterized by an extremely slow dissociation rate of GDP from the EF-Tu·GDP complex, whereas GTP is released from EF-Tu·GTP at a much faster rate (see Fig. 5). Determination of the dissociation rate constants (k_d') of the G-domain complexes with either GTP or GDP further emphasizes the differences between their properties and those of the corresponding complexes of EF-Tu. As illustrated in Fig. 5, the dissociation rates of the G-domain complexes are very close, whereas those of the EF-Tu complexes differ by ≈30 times. The properties of the G domain explain the efficient exchange of bound GDP with free GTP (data not illustrated), in contrast to the situation obtained with EF-Tu.

Of the EF-Tu effectors, elongation factor Ts (EF-Ts) and kirromycin appear to slightly retard the dissociation rates of G-domain·GTP and G-domain·GDP complexes (data not illustrated). In the intact molecule, EF-Ts unlocks the conformation of EF-Tu·GDP, strongly stimulating the dissociation and association rates of the EF-Tu·GDP complex (21, 22). This plays a crucial role in the regeneration of EF-Tu·GTP. In contrast to EF-Tu·GDP, the G-domain·GDP complex has an open conformation; this situation may possibly explain the different, slightly inhibitory effect of EF-Ts for both of the G-domain·guanine nucleotide complexes. The interaction of kirromycin with the intact molecule results in a strong inhibition of the dissociation rate of the EF-Tu·GTP complex and a stimulation of the dissociation rate for EF-Tu·GDP (22).

The G Domain Displays GTPase Activity. The presence of the catalytic center for GTP hydrolysis in the N domain of EF-Tu, suggested by the location of the GDP binding site, is now conclusively proven by the ability of the G domain to hydrolyze GTP. As shown in Fig. 6, the hydrolysis rate is linear over a long interval of time and corresponds to ≈0.1 mmol of GTP hydrolyzed per sec per mol of G domain, that

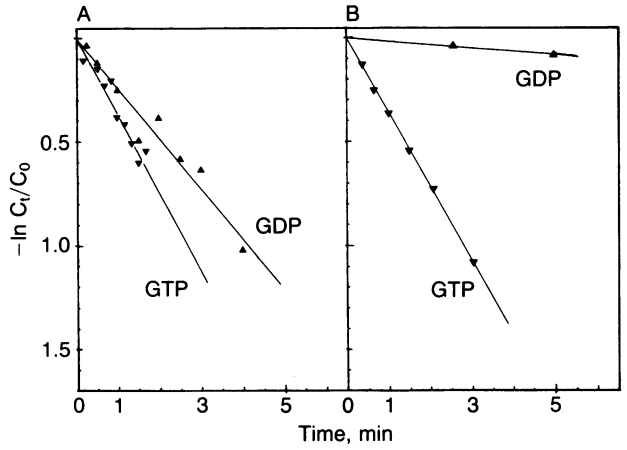

FIG. 5. Comparison of the dissociation rates (k_d) of the G-domain complexes with GTP and GDP (*A*) with those of the corresponding EF-Tu complexes (*B*). The dissociation rates of the G-domain complexes with GDP (▲) and GTP (▼) were determined in a reaction mixture containing, in 310 μl of standard buffer, 5.1 μM G domain, 5.3 μmol of [γ-^{32}P]GTP (specific activity, 1112 cpm·pmol^{-1}) or 2.9 μM G domain, and 4.1 μM [^{3}H]GDP (specific activity, 1950 cpm·pmol^{-1}). Complex formation occurred at 0°C during 30 min. To start the reaction, a 1000-fold excess of unlabeled GTP or GDP was added, and the solution was quickly mixed. At 30-sec intervals aliquots were withdrawn and filtered, and the radioactivity was measured. The half-life times for EF-Tu·GDP (▲) and EF-Tu·GTP (▼) complexes are reported from ref. 22. The calculated half-life times of the complexes were 118 sec for G domain·GTP and 166 sec for G domain·GDP, as compared to 117 sec for EF-Tu·GTP and 3120 sec for EF-Tu·GDP.

is in the range typical for the ras products (26, 27). The G-domain GTPase has been found to catalyze a multiple-round turnover reaction (data not illustrated). It is germane to mention that in the absence of effectors influencing its interaction with GDP/GTP, the initial rate of the single-turnover GTPase of EF-Tu·GTP (0.06 mmol of GTP hydrolyzed per sec per mol of EF-Tu) is very close to the rate of the multiple-round GTPase of the G domain (see above). In the case of EF-Tu the multiple turnover hydrolysis is strongly inhibited by the tightly bound product GDP (28, 29). The absolute specificity of EF-Tu for guanine nucleotides is conserved in the G domain; no detectable ATPase activity was found when tested using [γ-^{32}P]ATP of very high specific activity (data not shown).

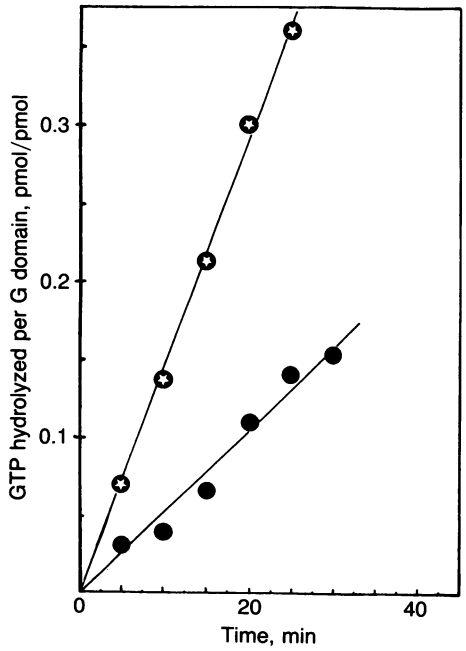

FIG. 6. GTPase activity of the G domain. The reaction mixture contained in a total volume of 300 μl of standard buffer, 0.8 μM G domain, 17 μM [γ-^{32}P]GTP (540 cpm·pmol^{-1}) and minus (solid circle) or plus (open star in solid circle) 0.8 μM 70S ribosomes. The reaction was started by the addition of [γ-^{32}P]GTP at 30°C. Aliquots of 40 μl were withdrawn at the intervals of time indicated. Liberation of P_i was carried out as described (18). The plotted values were corrected for the blank activity determined in the absence of G domain; in the absence of ribosomes the constant blank value was negligible (<1%) and ≈25% in their presence.

The ribosome is the most important positive effector of the EF-Tu GTPase, and aa-tRNA plays an essential role in coupling this reaction with the elongation process (18, 21). As shown in Fig. 6 ribosomes can still exert a significant stimulation on the G-domain-dependent GTPase. Of the other EF-Tu ligands, kirromycin and EF-Ts somewhat inhibit the reaction, and aa-tRNA was found to be inactive (data not shown). It is worth mentioning that in the presence of kirromycin, EF-Tu also displays a turnover GTPase in the absence of any other effector (30). This similarity is explained by the mode of action of the antibiotic, which partially relieves the differential effects of GDP and GTP on EF-Tu thus allowing an efficient exchange of the bound GDP for the free GTP (22). Under a similar ionic environment, the turnover GTPase of EF-Tu·kirromycin (31, 32) is about 15 times higher than that of the G domain without the antibiotic.

Concluding Remarks. The results presented in this article show that it is possible to isolate a functionally active G domain, representing the guanine nucleotide-binding domain of EF-Tu as determined by x-ray diffraction. The fact that this domain, when isolated, still conserves several of the functions of the intact EF-Tu molecule suggests that the removal of the Middle and C domain should not substantially affect its tertiary structure. The observed functional differences are most likely due to the relaxation of the control mechanisms exerted on the N domain by the Middle and C domains. The G domain is, therefore, useful for studying not only the basic activities of EF-Tu (such as guanine nucleotide interaction and GTPase activity), but also the functional role of the two other domains and the related allosteric mechanisms.

The most dramatic difference between the G domain and the intact molecule appears to be the inability of the former to distinguish between GTP and GDP. It can be concluded that the locked conformation induced by GDP on EF-Tu is relieved, the conformation of the guanine nucleotide-binding site of the G domain resembling the situation found with EF-Tu·GTP, the physiologically active complex. It is also striking that the initial hydrolysis rate of the single turnover of EF-Tu·GTP and the turnover rate of the G domain are very close. This indicates that removal of the Middle and C domains does not essentially affect the catalytic center. The ribosome is the only ligand that displays a clear influence on the GTPase of the G domain. The stimulation of the G-domain GTPase by ribosomes is a further demonstration of the important role of this ligand in inducing the GTP hydrolysis of EF-Tu. The aa-tRNA is inactive in this reaction whereas the inhibitory effects of EF-Ts and kirromycin appear to be of minor importance at least under the experimental conditions tested. Nevertheless, the fact that these two ligands interact with the G domain suggests that they may also have some kind of contact with the N domain of the intact molecule.

A series of common structural features relating the nucleotide binding domains of the guanine, adenine, and dinucleotide-binding proteins are apparent from homology and diffraction studies (6–11). A tentative model for the tertiary structure of the p21 ras protein has been proposed from the

crystallographic model of EF-Tu·GDP (33). In the protein p21, site-directed mutation of aspartic acid-119, the homologue of the EF-Tu residue aspartic acid-138 whose side chain is likely to interact with the guanine ring of GDP (6, 7) has resulted in a strong decrease of the affinity for GDP (34). Specific functions are suggested for several amino acid positions in EF-Tu and other guanine nucleotide binding proteins. According to primary sequence alignment, position 20 (valine) of EF-Tu corresponds to position 12 (glycine) of the ras product p21. The G domain would, therefore, correspond to the oncogenic variant of p21 having glycine-12 replaced by valine; this mutation enhances the transforming activity and reduces GTP hydrolysis (35, 36). Site-specific mutations of the G domain will be, therefore, a useful tool for investigating the structure–function relationships in this class of proteins.

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