Substitution of Aspartic Acid-80, a Residue Involved in Coordination of Magnesium, Weakens the GTP Binding and Strongly Enhances the GTPase of the G Domain of Elongation Factor Tu†

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ABSTRACT: The functional role of Asp80, a residue involved in the coordination of the Mg^{2+}·guanine nucleotide complex in elongation factor Tu (EF-Tu), has been investigated by its substitution with Asn in the isolated N-terminal domain (G domain). The G domain D80N is characterized by a strong decrease in binding affinity for GTP and magnesium, whereas the affinity for GDP is unchanged. This effect can be mimicked in wild-type G domain by the addition of EDTA. In contrast to this, EDTA does not essentially influence the selective effects of the mutation on the GTP and GDP binding of G domain D80N, indicating that the action of Asp80 is mainly mediated by the GTP-coordinated magnesium ion. The GTPase activity of the G domain D80N is very unstable, but can be markedly stabilized by the addition of glycerol without essentially modifying the specific effects of the mutation. In the absence of glycerol G domain D80N can express a short-lived GTPase activity. The presence of glycerol transforms this evanescent activity into a linear multiple-round activity that under optimal conditions can be almost 2 orders of magnitude higher than the GTPase of wild-type G domain. This enhanced catalytic activity represents the most striking consequence of the mutation and stresses the key role of Asp80 in the GTPase of EF-Tu. Our results strongly suggest that the catalytically active conformation of EF-Tu·GTP is favored by a relief of the constraints imposed by the interactions between Asp80, Thr25, and Mg^{2+} and that the activators of the EF-Tu GTPase (ribosomes, kirromycin, and monovalent cations) act by influencing the coordination of Mg^{2+}·GTP. This work illustrates the complexity of the structures controlling the binding and the cleavage of the γ-phosphate of GTP and emphasizes the central role of Asp80 in the GTPase activity and in maintaining the structural integrity of the G domain.

The GTPases comprise many families of proteins, which play regulatory roles in fundamental processes of the cell, such as proliferation and differentiation, protein biosynthesis, hormone response, neurotransmission, and the guidance of vesicular traffic within cells [for review see Bourne et al. (1990, 1991)]. GTP and GDP induce on these proteins two specific conformational states ("on" and "off", respectively) with different abilities to interact with macromolecules. Equilibria and binding rates of GTP and GDP, and the GTPase activity, modulate the functions of these proteins. Biochemical and genetic analysis of many GTPases indicates that they have a basic structure, conserved during evolution, and a switching mechanism with common characteristics but adapted to serve specific functions. Three-dimensional models derived from X-ray crystallography of two of these proteins, the bacterial elongation factor Tu (la Cour et al., 1985; Jurnak, 1985; Jurnak et al., 1989; Nyborg & la Cour, 1989; Kjeldgaard & Nyborg, 1992) and the human Ha-*ras* p21 protein (Pai et al., 1989; Milburn et al., 1990; Brünger et al., 1990; Tong et al., 1991), have confirmed these conclusions. A growing number of experimental observations indicate that residues in corresponding positions, even when nonconserved, mediate the same function in all GTPases [for references see Gümüsel et al. (1990)]; therefore, function–structure relationships derived from one of these proteins can be applied to the members of the other families.

The isolated N-terminal domain of EF-Tu[1] (G domain) binds GDP or GTP stoichiometrically and with comparable affinities, unlike the full-length EF-Tu that binds GDP 2 orders of magnitude more strongly than GTP. Therefore, due to a more favorable thermodynamic situation the turnover GTPase activity of the G domain is much higher than that of EF-Tu. These properties make the G domain a useful model to study the basic activities of EF-Tu, using site-directed mutagenesis (Cool et al., 1990; Gümüsel et al., 1990; Harmark et al., 1990, Cool & Parmeggiani, 1991).

Asp80 is the first residue of the second consensus element (Dever et al., 1987), which includes loop L4 and is conserved in all GTPases (Bourne et al., 1991). The three-dimensional model indicates that Asp80 is involved in the coordination of Mg^{2+}·GDP (Kjeldgaard & Nyborg, 1992). Since a wealth of data point to the importance of Mg^{2+} in GTPases, the substitution Asp80 → Asn has been carried out to assess the functional and structural implications of this residue and Mg^{2+}

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[1] Abbreviations: EF-Tu, elongation factor Tu from *Escherichia coli*; G domain wt, isolated N-terminal domain (residues 1–202) from wild-type EF-Tu; G domain D80N, mutant G domain in which Asp80 is replaced by Asn; loops L1, L2, and L4, the three EF-Tu loops corresponding topologically to p21 loops L1, L2, and L4, respectively.

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in EF-Tu. Our results support a key role of Asp80 in the coordination of the Mg^{2+}·GTP complex. Its substitution by Asn weakens the binding of this nucleotide, destabilizes the G domain, and as the most striking result induces a strong increase in the catalytic activity.

MATERIALS AND METHODS

Site-directed mutagenesis in the G domain of EF-Tu was carried out on a pEMBL9+ (Dente et al., 1983) carrying the truncated *tufA*(Δ610-1179) that encodes the G domain of EF-TuA. The oligodeoxynucleotide 3′-GCCCGTCAAATG CAC-5′ was the mutagenic primer used in the gapped duplex method (Parmeggiani et al., 1987). The mutated gene was overexpressed from the "runaway" vector pCP40 under control of λP_L (Remaut et al., 1983) in the *Escherichia coli* strain N4830 which contains a chromosomal thermosensitive λP_L repressor (Gottesman et al., 1980). A 14-L culture (LB medium with 100 mg/L ampicillin) was grown at 28 °C in a 20-L fermentor (Biolafitte) to a cell density of 0.2 A_{600} units, at which point the overproduction was induced by rapidly raising the temperature to 42 °C. After 1-h incubation at 42 °C followed by 1-h incubation at 38 °C, 12 g of cells were harvested by centrifugation and resuspended (1 g/10 mL) in buffer A [50 mM Tris·HCl, pH 7.65, 100 mM KCl, 10 mM $MgCl_2$, 7 mM 2-mercaptoethanol, 1 mM phenylmethanesulfonyl fluoride, 40 μM GDP, and 15% (v/v) glycerol]. After sonication, the cell extract was centrifuged for 60 min at 30000*g* and 4 °C. The resulting supernatant was dialyzed against buffer B (as buffer A except for 25 mM KCl) and then applied to a DEAE-Sepharose Fast Flow (Pharmacia) column (1.5 cm × 75 cm) using a linear KCl gradient (25–200 mM, total volume 3 L) in a buffer as A except for KCl. The fractions containing the G domain were eluted between 105 and 115 mM KCl in a 150-mL volume, and after dialysis against buffer A, they were concentrated in Aquacide II and redialyzed against buffer A. The concentrate (3.5 mL) was passed through a long Ultrogel AcA54 (IBF) column (2 × 196 cm) in buffer A at a rate of 15 mL/h. The G domain D80N emerged at 345–385 mL. The central part of the active peak was 99% pure as judged from SDS/PAGE. After concentration and dialysis against buffer C [50 mM Tris·HCl, pH 7.65, 50 mM KCl, 10 mM $MgCl_2$, 7 mM 2-mercaptoethanol, 40 μM GDP, and 50% (v/v) glycerol], the G domain D80N was stored at −20 °C.

Determination of the apparent dissociation constants (K'_d) was performed using the nitrocellulose filtration method (Jensen et al., 1989). The GTPase activity was measured by the charcoal method (Ivell et al., 1981) and protein concentration by the Bradford method (1976) using bovine serum albumin as standard. For more technical details, see legends to figures and Harmark et al. (1990).

RESULTS

Overproduction and Purification of G Domain D80N. Overexpression of the plasmid-borne mutated *tufA* affects the host cell, leading progressively to a nearly complete inhibition of cell growth after induction. Nevertheless, the overproduced G domain D80N represents up to 20% of the total cell protein. Depending on the preparation, the soluble portion varies from 20 to 30% after centrifugation of the cell extract for 1 h at 30000*g* at 4 °C, which is somewhat less than the G domain wt overproduced under the same conditions. FPLC chromatography, a technique which has been successfully applied to the purification of the G domain and several of its mutants (Parmeggiani et al., 1987; Harmark et al., 1990; Cool et al., 1990), yielded a homogeneous G domain D80N but deprived of any activity (not illustrated). The possibility that substitution of this important residue, involved in the binding of Mg^{2+} and in the coordination of loop 82–83 with loop 18–23 (Woolley & Clark, 1989; Kjeldgaard & Nyborg, 1992), had destabilized the G domain prompted us to replace FPLC with a milder procedure at atmospheric pressure, such as ionic chromatography on DEAE-Sepharose Fast Flow and filtration on Ultrogel AcA54.

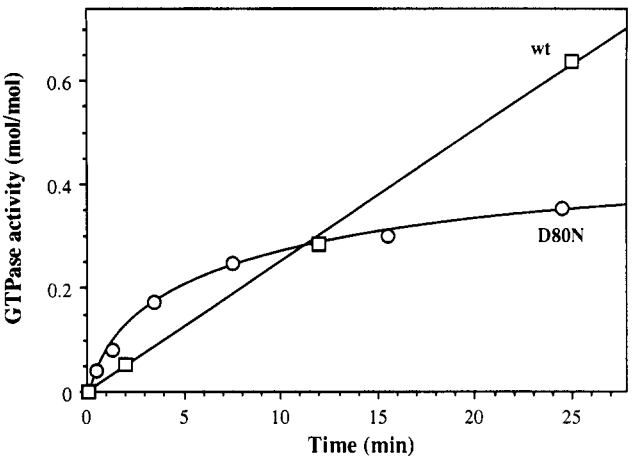

FIGURE 1: Kinetics of the GTPase activity of G domain wt and D80N in the absence of glycerol. The reaction mixtures (500 μL), containing 50 mM imidazole acetate, pH 7.65, 1 M KCl, 0.1 mM $MnCl_2$, 1 mM dithiothreitol, 100 μM [γ-^{32}]GTP (425 cpm·pmol^{-1}), and 2 μM G domain wt (□) or D80N (○), were incubated at 20 °C. At the indicated times samples (60 μL) were withdrawn and the liberated [^{32}P]P_i was determined.

Mutation D80N Strongly Enhances the GTPase Activity. We have carried out all our assays on the GTPase activity of the G domain D80N at 20 °C, since in previous work concerning the rather unstable G domain E117Q this temperature was better tolerated than 30 °C (Harmark et al., 1990). Still, even at 20 °C the catalytic activity of the G domain D80N was detectable only at 0.1 mM $MnCl_2$ and 1 M KCl, i.e., under ionic conditions found to induce optimal GTPase activity on G domains wt, Q114E, and E117Q (Harmark et al., 1990). We were thus able to trace a G domain D80N-dependent GTPase; however, this activity, unlike that of G domain wt, decreased rapidly (Figure 1). To reduce this pronounced instability, we used glycerol during the assay. Indeed, the presence of 20 or 40% glycerol allows a turnover activity persisting for several hours and comprising multiple rounds of GTP hydrolysis. Figure 2 shows that the extent and linearity of this activity increase with increasing concentrations of glycerol. These results led us to determine the GTPase activity as a function of glycerol concentration in the presence of 200 mM and 1 M KCl, respectively (Figure 3, panels A and B). Under these conditions, the optimum activity was obtained around 45–55% glycerol. It is important to mention that, at a glycerol concentration around 40%, the velocity of the G domain D80N GTPase was close to the initial velocity observed in the absence of this stabilizer [0.2–0.5 mol of GTP hydrolyzed·(mol of G domain D80N)$^{-1}$·min^{-1}, depending on the assay conditions]. Unlike G domain D80N, the G domain wt GTPase was little stimulated by glycerol while the activity of EF-Tu·kirromycin, which was almost unaffected up to 25% glycerol, became progressively inhibited at higher concentrations (Figure 3A). Taken together, these observations strongly support the view that glycerol stabilizes the G domain D80N and only indirectly enhances its catalytic activity. At optimum glycerol concentrations the GTPase activity of G domain D80N is more than 80 times higher than

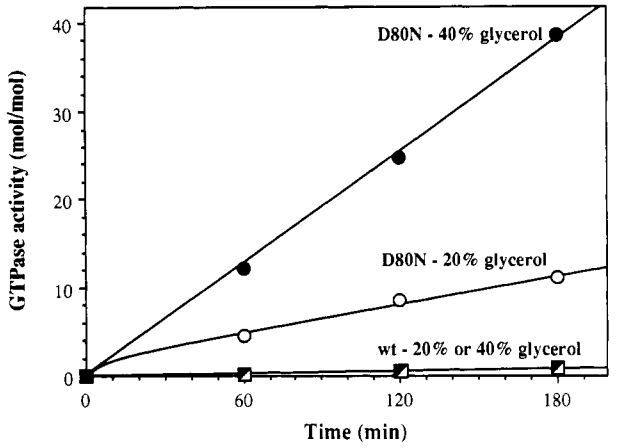

FIGURE 2: Kinetics of the GTPase activity of G domain wt and D80N in the presence of glycerol. The reaction mixtures (300 μL), containing 50 mM imidazole acetate, pH 7.65, 10 mM $MgCl_2$, 200 mM KCl, 1 mM dithiothreitol, 1 μM G domain wt (□, ■) or G domain D80N (○, ●), 10 μM (in the case of G domain wt) or 160 μM (in the case of G domain D80N [γ-^{32}P]GTP (150–1500 cpm·pmol^{-1}), and 20% (open symbols) or 40% (closed symbols) glycerol (v/v) were incubated at 20 °C. At the indicated times 50-μL samples were withdrawn and the liberated [^{32}P]P_i was determined.

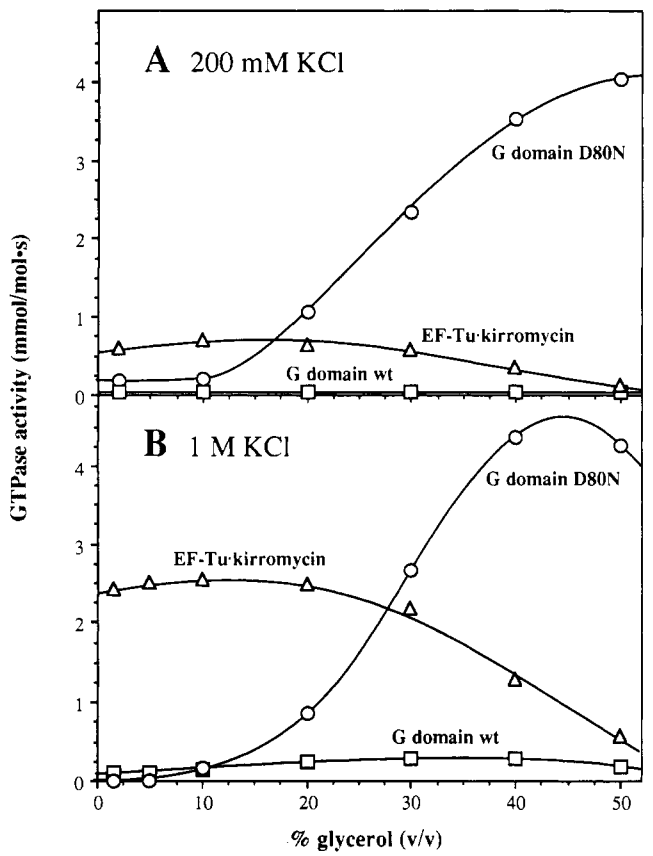

FIGURE 3: The concentration effect of glycerol on the GTPase activity of G domain wt and D80N, and of EF-Tu·kirromycin. The reaction mixtures (60 μL) contained 50 mM imidazole acetate, pH 7.65, 10 mM $MgCl_2$, 200 mM KCl (A) or 1 M KCl (B), 1 mM dithiothreitol, 2 mM phosphoenolpyruvate, 50 ng/μL pyruvate kinase, 1 μM G domain wt (□), G domain D80N (○), or EF-Tu·kirromycin (△), and 10 μM (in the case of G domain wt) or 160 μM (in the case of G domain D80N and EF-Tu·kirromycin) [γ-^{32}P]GTP (150–1500 cpm·pmol^{-1}). After 160 min of incubation at 20 °C, the liberated [^{32}P]P_i was determined.

that of G domain wt and even 5–6 times higher than the activity of EF-Tu·kirromycin. Increasing the KCl concentration from 0.2 to 1 M has little effect on the GTPase activity of G domain D80N, while, as expected, this markedly increases the GTPase of the G domain wt and even more that of EF-Tu·kirromycin [Figure 3B; cf. Ivell et al. (1981) and Harmark et al. (1990)]. Notably, glycerol inhibits the intrinsic GTPase of EF-Tu already at concentrations higher than 10% (not illustrated).

Table I: K_m, k_{cat}, k_{cat}/K_m and Change in Free Energy of Activation of the GTPase Activity of G Domain D80N and wt[a]

	K_m (μM)	k_{cat} ($s^{-1}\cdot10^3$)	k_{cat}/K_m ($s^{-1}\cdot M^{-1}$)	$\Delta(\Delta G^{\ddagger})$ (kJ·mol^{-1})
G domain wt	0.84	0.06	71	
G domain D80N	16.0	3.65	228	−2.8

[a] 1 μM enzyme was incubated for 110 min at 20 °C with different concentrations of [γ-^{32}P]GTP (2–120 μM specific activity 165 cpm·pmol^{-1}) in a buffer containing 10 mM $MgCl_2$, 200 mM KCl, 50 mM imidazole acetate, pH 7.65, 1 mM dithiothreitol, 2 mM phosphoenolpyruvate, 50 ng/μL pyruvate kinase, and 35% glycerol. The liberated [^{32}P]P_i was determined by the charcoal method. The K_m and k_{cat} values were calculated from the obtained data using Lineweaver–Burk plots. The apparent contribution [$\Delta(\Delta G^{\ddagger})$] of the mutated side chain to the binding energy of the enzyme–transition state complexes was calculated by comparing the ratios of k_{cat}/K_m for activation by wild-type and mutant enzyme, using the equation $\Delta(\Delta G^{\ddagger}) = -RT\ln\{(k_{cat}/K_m)_{D80N}/(k_{cat}/K_m)_{wt}\}$.

In the following experiments, glycerol was present at a 35% concentration. This is sufficient for a reasonable stabilization of the enzyme and low enough to avoid effects of this solvent on the catalytic activity (see Discussion).

In the presence of 10 mM $MgCl_2$, 200 mM KCl, and 50 mM imidazole acetate, pH 7.6, the k_{cat} of the G domain D80N is 3.65 mmol of GTP hydrolyzed·(mol of enzyme)$^{-1}\cdot s^{-1}$, i.e., 60 times higher than that of the G domain wt [0.06 mmol of GTP hydrolyzed·(mol of enzyme)$^{-1}\cdot s^{-1}$] and 6 times the activity of EF-Tu·kirromycin in the absence of glycerol [0.6 mmol of GTP hydrolyzed·(mol of enzyme)$^{-1}\cdot s^{-1}$] (Figure 3A and Table I). The K_m of G domain D80N GTPase is 20 times higher than that of the G domain wt (16 vs 0.84 μM, Table I), while the k_{cat}/K_m ratio shows that the efficiency of the reaction has increased 3 times. The change in free energy of activation [$\Delta(\Delta G^{\ddagger})$] induced by the mutation has been found to be −2.8 kJ mol^{-1}, a value that lies at the lower range of a weak hydrogen bond and that may be not significant.

It is important to mention that, even in the presence of 50% glycerol, the GTPase activity of the G domain D80N shows a constant decline upon storage at −20 °C. Its half-life depends on the preparation and varies from 1 to 3 weeks (not shown).

Effect of Monovalent and Divalent Cations on the GTPase of G Domain D80N. We have studied the concentration effects of diverse monovalent and divalent cations, taking as a reference the G domain wt. As shown in Figure 4A, in the presence of glycerol the G domain wt GTPase increases in a nearly linear fashion up to a 1.5 M concentration of monovalent salts. K^+ induces the highest activity, closely followed by NH_4^+, Na^+, and Li^+, which display virtually the same effect. The substitution Asp80 → Asn considerably influences the concentration effects of the monovalent cations: K^+ and NH_4^+ induce a saturation type curve on the G domain D80N GTPase while the specific differences between the cations become much more pronounced (Figure 4B). The order of effectivity is $K^+ > NH_4^+ > Na^+ > Li^+$, i.e., the same as found for G domain Q114E (Harmark et al., 1990). Since crystallographic data indicate that Asp80 and Gln114 are both involved in interactions with loop L1 (residues 18–23) via the side chain of Thr25 and the main-chain amide of Val20, respectively, these results support our earlier suggestions that the path of activation by monovalent cations specifically concerns this region (Harmark et al., 1990).

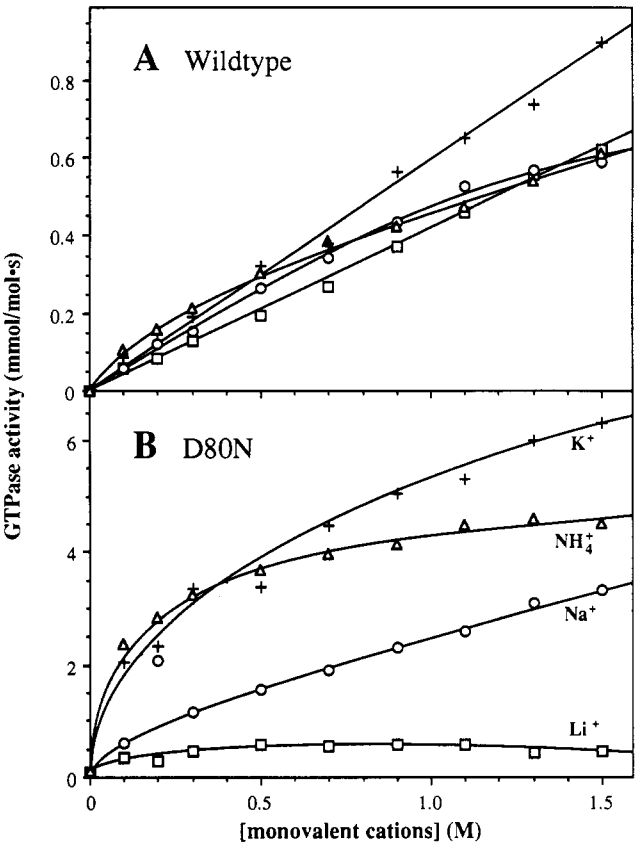

FIGURE 4: The effect of different monovalent cation species on the GTPase activity of G domain wt (A) and D80N (B). Reaction mixtures (60 μL) containing 50 mM imidazolium acetate, pH 7.65, 0.5 mM $MnCl_2$, 1 mM dithiothreitol, 35% glycerol, 160 μM [γ-^{32}P]GTP (100 cpm·pmol^{-1}), and 1 μM G domain were incubated in the presence of the indicated concentrations of monovalent cations at 20 °C for 100 min, and the liberated [^{32}P]P_i was determined. KCl (+), LiCl (□), NaCl (○), and NH_4Cl (△).

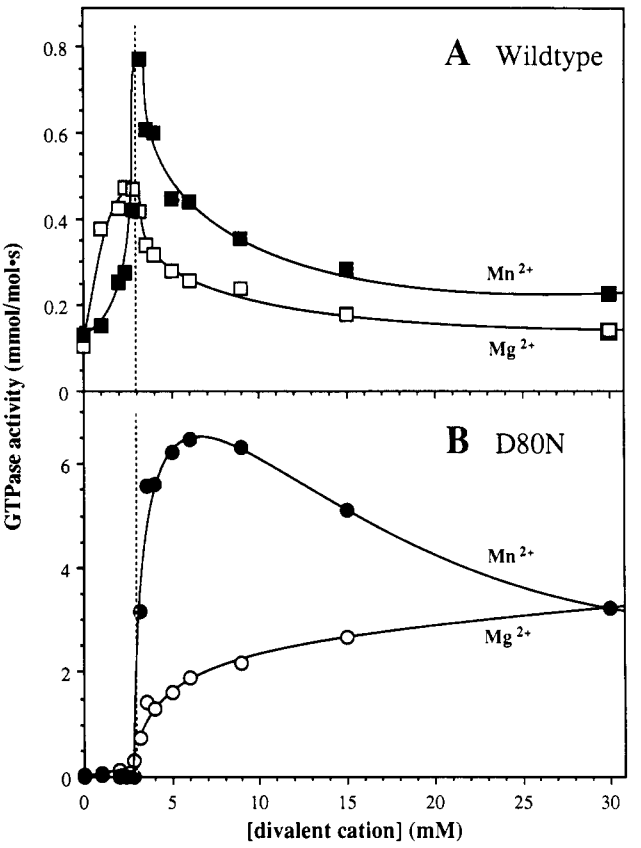

FIGURE 5: The effects of different divalent cation species on the GTPase activity of G domain wt (A) and D80N (B). Reaction mixtures (60 μL) containing 50 mM imidazolium acetate, pH 7.65, 1 M KCl, 1 mM dithiothreitol, 3 mM EDTA, 35% glycerol, 160 μM [γ-^{32}P]GTP (100 cpm·pmol^{-1}), and 1 μM G domain wt (□, ■) or D80N (○, ●) were incubated in the presence of the indicated concentrations of Mg^{2+} (○, □) or Mn^{2+} (■, ●) at 20 °C for 100 min, and the liberated [^{32}P]P_i was determined. The dotted line indicates the concentration of EDTA.

Also the concentration effect of Mg^{2+} on the G domain D80N GTPase is markedly modified by the mutation: from a sharply defined optimum to a saturation type curve [Figure 5; cf. also Harmark et al. (1990)]. Concerning the Mn^{2+} effect, the optimum concentration range has become broader and is shifted from 0.1 to 3–4 mM. Moreover, taken together, panels A and B of Figure 5 show that Mg^{2+} and Mn^{2+} are less tightly bound to G domain D80N than to G domain wild type. In fact, in the latter case the presence of >3 mM EDTA is required for a full inhibition of the GTPase activity, whereas for the mutant a concentration of <1 mM EDTA is sufficient.

It is worth mentioning that the presence of free Mg^{2+} even in very low concentrations is an absolute requirement for the GTPase activity of EF-Tu, as well as for other small GTP-binding proteins such as Ha-*ras* p21 (Mistou et al., 1992). In the case of the GTPase activity of EF-Tu·kirromycin, it was found that at least 10 mM EDTA is needed to completely inhibit the catalytic reaction (Ivell et al., 1981).

Mutation of Asp80 Selectively Affects the Binding of GTP. In experiments not illustrated, we have observed that, upon storage in 50% glycerol for several weeks, the G domain D80N can bind GDP nearly stoichiometrically [0.93 mol of GDP·(mol of protein)$^{-1}$] also without glycerol in the assay system, despite the pronounced instability of the catalytic activity. As shown in Table II, the binding affinity for GDP is about the same as that of G domain wt. As opposed to this, the affinity for GTP is reduced to such an extent [K'_d(GTP) = 92 μM versus 4 μM for the G domain wt] that it can only be measured indirectly via competition with GDP, at least using the nitrocellulose binding assay. Addition of glycerol (35%) does not essentially modify this picture.

Table II: Effect on the GDP/GTP Affinities by the Substitution Asp80 → Asn in the G Domain of EF-Tu[a]

complexes	K'_d (μM) no glycerol	K'_d (μM) 35% glycerol
G domain wt·GDP	2.2[b]	1.1
G domainD80N·GDP	2.3	1.5
G domain wt·GDP + EDTA	5.4	1.0
G domainD80N·GDP + EDTA	2.7	1.6
G domain wt·GTP	4.0[b]	3.8
G domainD80N·GTP	92	40
G domain wt·GTP + EDTA	481	17
G domainD80N·GTP + EDTA	254	37

[a] The K'_d values of the GDP complexes were determined by incubating 1 μM enzyme at 0 °C with different concentrations of [^{3}H]GDP (1–30 μM, specific activity 100 cpm·pmol^{-1}) in 60 μL of buffer containing 10 mM $MgCl_2$, 200 mM KCl, 50 mM imidazole acetate, pH 7.65, plus or minus 17 mM EDTA, 1 mM dithiothreitol, and 35% glycerol. At equilibrium an aliquot of 50 μL was applied onto a nitrocellulose filter and washed by two times 3 mL of ice-cold buffer. The K'_d values of the GTP complexes were determined in competition experiments. [b] Values from Harmark et al. (1990).

The differences in behavior between the GTP and GDP complexes of G domain wt and D80N are particulary evident in the presence of a quantity of EDTA sufficient to sequestrate any coordinated Mg^{2+} in the G domain (100 mM EDTA was used; K. Harmark, unpublished). As shown in Table II, at this condition there is little change, if any, in the K_d of the

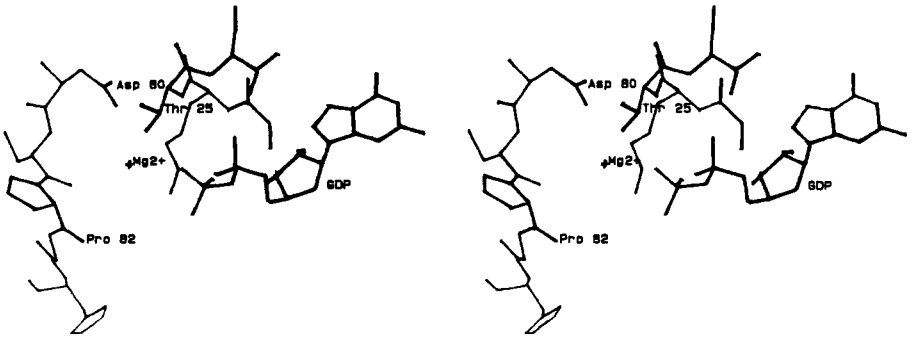

FIGURE 6: Stereo diagram of the structural model of the guanine nucleotide binding pocket of the EF-Tu·GDP complex, illustrating the environment of Asp80.

GDP complex of both G domain wt and D80N, whether or not glycerol is present. In contrast to this, the properties of the GTP complex of G domain wt are markedly changed and closely resemble those of G domain D80N·GTP, which in comparison are little affected by the presence of EDTA. These results show that with G domain wt EDTA can mimic the effect of the mutation as concerns the GTP complex.

DISCUSSION

In GTPases divalent cations are essential for proper interaction with the nucleotide, catalytic activity, and structural integrity of the molecule. In proteins, Mg^{2+} and Mn^{2+} ions are coordinated by six interactions in an octahedral arrangement (Eccleston et al., 1981; Williams, 1983; Einspahr & Bugg, 1984; Brown, 1988). EPR, NMR, and PRR studies (Wilson & Cohn, 1977; Eccleston et al., 1981; Wittinghofer et al., 1982; Kalbitzer et al., 1984; Rösch et al., 1986) indicate that in EF-Tu·GDP three of these interactions concern water molecules while the remaining interactions concern a β-phosphate oxygen of GDP and amino acid residues. The available three-dimensional model at medium resolution of trypsinized EF-Tu·GDP gives only partial information about the coordination of Mg^{2+} (Figure 6), since it does not allow the identification of water molecules (Kjeldgaard & Nyborg, 1992). However, useful information can be obtained from the model of p21·GDP at high resolution, since the overall α/β topology of the GDP binding domains of these two proteins is identical and more than 80% of the polypeptide backbones are superposable, in particular around the substrate binding site (Pai et al., 1990; Jurnak et al., 1990; Tong et al., 1991; Valencia et al., 1991). Thus in EF-Tu, Mg^{2+} appears to interact directly with the β-phosphate and Thr25, and indirectly with Asp80 and the main-chain carbonyl of Pro82, likely via water molecules. The side chains of Thr25 and Asp80 very probably interact though a hydrogen bond; therefore, one can deduce that EF-Tu has a chelation ring (Asp80–H_2O–Mg^{2+}–Thr25–Asp80) similar to that described for p21·GDP and p21·GppNp or p21·GTP (Asp57–H_2O–Mg^{2+}–Ser17–Asp57; Pai et al., 1990; Tong et al., 1991).

According to the p21·GppNp model, the change in Mg^{2+} coordination upon binding of GTP affects three free water molecules: one is replaced by the γ-phosphate of GTP, a second by the side-chain hydroxyl group of Thr35, whose main-chain amide moiety forms a hydrogen bond with one of the γ-phosphate oxygens, and a third becomes bonded to the main-chain carbonyl of Asp33. The interactions with the remaining ligands (the chelation ring and the β-phosphate) are conserved (Pai et al., 1990; Tong et al., 1991). Therefore, from the p21 models one can deduce that in EF-Tu·GTP (A) the interactions of Asp80 and Thr25 with Mg^{2+} are not affected, (B) Thr61 of the effector region (corresponding to Thr35 in p21 and conserved in all GTPases; Bourne et al., 1991) replaces one of the water molecules coordinated to Mg^{2+}, and (C) a second water molecule coordinated to Mg^{2+} is substituted by the γ-phosphate.

Asp80 → Asn is the first mutation found to enhance the catalytic activity of the G domain. Since this replacement weakens the chelation of Mg^{2+}, our results suggest that the γ-phosphate cleavage involves a relief of the constraints imposed by Asp80 on the Mg^{2+}·GTP coordination. A direct intervention on the γ-phosphate by Asn80 is improbable, at least from the present model. So far, the existence of a catalytic residue in EF-Tu has not been established. The possibility that His84, the most obvious candidate, participates in a nucleophilic attack on the γ-phosphate by activation of a water molecule, as proposed for the corresponding Gln61 in p21 (Pai et al., 1990), remains an open question (Cool & Parmeggiani, 1991). His84 is probably one of many residues involved in the stabilization of the transition state, as part of a mechanism that can constitute a major factor for the intrinsic catalysis [cf. Fersht et al. (1988)]. Metal ions can favor the phosphate transfer by indirect and direct mechanisms (Cooperman, 1976, 1982). A direct mechanism is likely to operate in enzymatic systems (Sigel & Hofstetter, 1983; Sigel et al., 1984). In EF-Tu, this would involve a nucleophilic attack by a Mg^{2+}-bound hydroxide in an intramolecular fashion, implying a pentacoordinate transition state. Since the cleavage of the γ-phosphate in EF-Tu occurs via an in-line transfer mechanism (Eccleston & Webb, 1982), a candidate for activation could be the water molecule connecting the main-chain carbonyl of Pro82 to Mg^{2+}.

The intrinsic GTPase activity of EF-Tu is very low but can be dramatically increased (5 orders of magnitude) by mRNA-programmed ribosomes and to a minor extent (1–2 orders of magnitude) by other ligands, such as vacant ribosomes, the antibiotic kirromycin, and monovalent cations. These effectors could stabilize the active conformation, or accelerate a conformational change preceding and limiting the hydrolytic step, or in the case of ribosomes also act as donor of a nucleophilic charge [for references, see Bourne et al. (1991)]. It is possible that the slow intrinsic GTPase is supported, at least in part, by mechanisms different from those underlying the burst-like catalysis taking place during polypeptide synthesis, and that may result from a combination of diverse effects. Independently of the cleavage mechanism, the results obtained with the G domain D80N indicate that the coordination between Asp80, Thr25, and Mg^{2+} is actively involved in EF-Tu catalysis.

Our results show that G domain D80N binds Mg^{2+} more weakly than G domain wt and that Mg^{2+} selectively influences the binding of GTP. The ability of EDTA to mimic the effects of the substitution Asp80 → Asn proves that the changes induced by the mutation are mediated by Mg^{2+}. Notably, in

G proteins with the same activation dependency as the G domain, the binding of GTP alone results in a small conformational change as compared to a major change if GTP is bound in the presence of Mg^{2+} (Higashijima et al., 1987a,b). However, even though the G domain and EF-Tu can still bind GTP in the presence of EDTA, their GTPases are strictly dependent on Mg^{2+} (Ivell et al., 1981; Jensen et al., 1989; Mistou et al., 1992; K. Harmark, unpublished results).

Since the initial velocity of GTP hydrolysis by G domain D80N is not essentially affected by glycerol, this agent appears to act as a stabilizer of the native conformation. Gekko and Timasheff (1981a,b) have shown for several proteins that glycerol in aqueous solutions, at least up to 40%, merely induces a preferential hydration of the protein, favoring a more folded and native state of the molecule. That glycerol is not responsible for the increased catalytic activity of the G domain D80N is also shown by the little effect of the latter on the GTPase of G domain wt.

The observation that chelation of Mg^{2+} by EDTA reduces the affinity for GTP more strongly in the absence of glycerol confirms that the overall integrity of the G domain is important for the binding of this nucleotide. As deduced from the p21 model, in EF-Tu this effect should be mediated by loop L4 via Thr61, and possibly by loop L1 that is wrapped around the phosphate groups. The network of crucial interactions, in which Asp80 is involved, explains the importance of this residue for the structural and functional integrity of this enzyme. Strikingly, the GDP binding is not at all influenced by the removal of Mg^{2+}. Unlike this, in intact EF-Tu EDTA strongly influences the binding of GDP (Miller & Weissbach, 1977), showing that the middle and C-terminal domains are indispensable for the tight conformation of the EF-Tu·Mg^{2+}·GDP complex.

In conclusion, this work illustrates the multiple functions of Asp80 and reveals the possibility of increasing the GTPase activity of EF-Tu by the substitution of a single residue involved in the coordination of the substrate-bound magnesium.

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