Substitution of Histidine-84 and the GTPase Mechanism of Elongation Factor Tu†

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ABSTRACT: Mutation of His84, a residue situated in one of the loops forming the guanine nucleotide binding pocket, was introduced in the G domain, the isolated N-terminal half molecule of bacterial elongation factor Tu (EF-Tu), in order to investigate the role of this residue on the basic activities of EF-Tu: the interaction with GDP and GTP and the hydrolysis of GTP. Substitution of His84 by Gly reduces the GTPase activity of the G domain to 5%; this activity can still be stimulated by raising the KCl concentration as the activity of wild-type G domain or the intact molecule. Since the affinities of the mutant protein for GDP and GTP are essentially the same as those of the wild-type G domain, His84 is apparently not involved in the binding of the substrates. Calculations of the change in free energy of activation of the GTPase reaction following substitution of His84 by Gly point to the disruption of a weak hydrogen bond, involved in the catalytic reaction. This probably concerns an interaction via a water molecule. The possible mechanism underlying the GTPase reaction is discussed in light of the three-dimensional structure of EF-Tu, taking into account the situation of Ha-*ras* p21.

The large family of guanine nucleotide binding proteins (Woolley & Clark, 1989) contains only two members with a crystal structure elucidated at high resolution (Jurnak, 1985; la Cour et al., 1875; Pai et al., 1989; Milburn et al., 1990): elongation factor Tu (EF-Tu),[1] an essential component of protein biosynthesis (Miller & Weissbach, 1977), and Ha-*ras* product p21, mutants of which have been found to be involved in the malignant transformation of the human cell (Barbacid, 1987). Both proteins have been extensively studied with respect to their functional activity, in particular of their intrinsic GTPase activity. GTP hydrolysis is a key reaction for the GTP binding proteins and represents the switching-off mechanism of their specific pathway, since these proteins are no longer active in the GDP-induced conformation. Kirromycin, a highly specific antibiotic increasing the intrinsic GTPase activity of EF-Tu, has proved to be a useful tool for the characterization of this catalytic activity (Chinali et al., 1977; Parmeggiani & Swart, 1985). The stereochemistry of the GTPase reaction of EF-Tu associated with kirromycin strongly suggests that the terminal phosphate of GTP undergoes a nucleophilic attack by a water molecule (Ecclestone & Webb, 1982). Taking into account the mechanism of DNase I as suggested by Suck and Oefner (1986) and the three-dimensional model of EF-Tu, Swart (1987) proposed for the GTPase of EF-Tu a mechanism based on the transfer of a proton from a water molecule via His84 to Glu117.

To shed more light on the interaction with the guanine nucleotides and the GTPase mechanism, we have substituted His84 with glycine. This mutation has been carried out in the gene encoding the GTP binding domain corresponding to the N-terminal half of the EF-Tu molecule (Parmeggiani et al., 1987). The truncated EF-Tu, designated the G domain, has been shown to bind GDP and GTP and catalyzes efficiently the hydrolysis of GTP (Jensen et al., 1989), without being exposed to the allosteric controls present in the intact molecule. It is therefore a suitable tool to investigate the basic properties of these activities. The results presented in this paper highlight the importance of His84 for the intrinsic GTPase activity of EF-Tu and allow discussion of possible alternatives for its role in the molecular mechanism underlying GTP cleavage.

Materials and Methods

Plasmids and *Escherichia coli* strains were kindly provided by Drs. R. Cortese (pEMBL9$^+$, EMBL, Heidelberg), W. Fiers (pCP40, pcI857; University of Gent), and L. Bosch (PM455; University of Leiden).

Site-directed mutagenesis was carried out as described earlier (Parmeggiani et al., 1987; Cool et al., 1989), using a pEMBL9$^+$ on which *tufA*(Δ610-1179) was cloned and the oligodeoxynucleotide 5′-CCG-GGG-*GG*C-GCC-GAC-3′ as the mutagenic primer. The mutated gene was transferred to the "runaway" vector pCP40 under control of λP_L (Remaut et al., 1983). For overproduction, *E. coli* strain PM455 (Van der Meide et al., 1983) was used, containing pcI857 which expresses the thermosensitive repressor of λP_L (Remaut et al, 1983). After determination of the optimum conditions for overproduction of soluble G domain, a culture was grown at 28 °C in a 20-L fermentor (Biolafitte) up to a cell density of 0.2 A_{600} unit, at which point the temperature was rapidly increased to 42 °C. After an additional incubation of 2 h, the cells were harvested, sonicated, and centrifuged as described (Parmeggiani et al., 1987). Purification of the overproduced, mutated G domain was carried out in three chromatography steps: DEAE-Sepharose Fast Flow (Pharmacia), Ultrogel AcA54 (IBF), and finally MonoQ, using the fast protein liquid chromatography system of Pharmacia. The gel filtration step was repeated in order to remove all traces of EF-Tu and elongation factor G.

Protein concentration was determined by the method of Bradford (1976), using bovine serum albumin as a standard. Apparent dissociation constants and dissociation rates were determined in standard buffer (50 mM imidazole acetate, pH 7.5-7.7, 50 mM NH_4Cl, 10 mM $MgCl_2$, and 1 mM dithiothreitol) by the nitrocellulose filtration method (Jensen et al., 1989). The association rate constants were calculated by the equation: $K_d' = k_{-1}'/k_{+1}'$. The GTPase activity was measured by the liberation of inorganic phosphate, using the isopropyl

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[1] Abbreviations: EF-Tu, elongation factor Tu from *Escherichia coli*; G domain, isolated N-terminal domain of EF-Tu; GTPase, guanosine-5′-triphosphatase.

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Table I: Effect of Substitution of His84 by Gly on GDP/GTP Interactions of the G Domain[a]

complexes	dissociation constant, K_d' (μM) 0 °C	30 °C	association rate constant, k_{+1}' ($M^{-1}s^{-1}$)[b] at 0 °C	dissociation rate constant, k_{-1}' (s^{-1}) at 0 °C	dissociation half-time (s) at 0 °C
G domain·GDP[c]	2.1	3.0	1.3×10^3	2.8×10^{-3}	248
G domain HG84·GDP	2.5	5.0	0.9×10^3	2.3×10^{-3}	300
G domain·GTP	3.6	9.0	1.8×10^3	6.3×10^{-3}	110
G domain HG84·GTP	4.1	8.3	1.0×10^3	3.9×10^{-3}	178

[a] The K_d' values were determined by incubating 1.0–1.5 μM protein in 35 μL of standard buffer containing 0.8–14 μM [^{3}H]GDP (specific activity 500 cpm/pmol) or 0.7 μM protein in 50 μL of standard buffer containing 2–40 μM [γ-^{32}P]GTP (specific activity 970 cpm/pmol). At equilibrium (5 min at 30 °C or 20–30 min at 0 °C), an aliquot of respectively 30 or 40 μL was filtered onto nitrocellulose, followed by a washing with 3 mL of ice-cold standard buffer. Dissociation rates were measured in 310 μL of standard buffer by incubating 2.8–5.5 μM protein with [^{3}H]GDP (specific activity 1300 cpm/pmol) or [γ-^{32}P]GTP (specific activity 1800 cpm/pmol) for 20 min at 0 °C, whereafter a 1000-fold excess of nucleotide was added. Aliquots of 20 μL were taken at different time intervals and filtered onto nitrocellulose, followed by a washing of 3 mL of ice-cold standard buffer. [b] Calculated according the equation: $k_{+1} = k_{-1}/K_d$. [c] Taken from Jensen et al. (1989).

acetate/molybdate method (Parmeggiani & Sander, 1981) or the charcoal method (Crêchet & Parmeggiani, 1986). For further details, see legends to tables and figures.

Results

Purification and General Properties of G Domain HG84. The mutated product was expressed in the *E. coli* cell as effective as the wild-type G domain (not shown) and was overproduced up to 20% of the total proteins of the cell extract. No anomalies in the growth rate of the cells were seen. G domain HG84 remained 50% soluble after centrifugation at 150000*g* for 150 min. After the three purification steps mentioned under Materials and Methods, the mutant protein was at least 99% pure, and its yield was approximately 0.5 mg of protein·(g of cell)$^{-1}$. Neither proteolytic nor aggregation phenomena were observed during and after purification. The mutant protein was stored in 25 mM Tris·HCl, pH 7.7, 50 mM NH_4Cl, 10 mM $MgCl_2$, 20 μM GDP, 7 mM 2-mercaptoethanol, and 50% glycerol. Under these conditions, it conserved the same level of guanine nucleotide binding and GTPase activity for at least 6 months when kept at −20 °C. The GDP bound by the different preparations of G domain HG84 was 0.8–1.0 mol/mol, as referred to the protein determination by the Bradford method. The stability of the protein against temperature-induced denaturation was determined in standard buffer as described by Jensen et al. (1989); for both the wild-type and the mutated G domain, the temperature at which 50% of the protein was no longer active in GDP binding was 39 °C (not shown).

Interaction with GDP and GTP. In Table I we report the apparent dissociation constants and the dissociation rate constants of the complexes with GDP and GTP of G domain HG84 as compared to wild-type G domain. Apparently, the substitution of His84 by Gly hardly affects the affinities for GDP or GTP, as shown by the nearly identical dissociation constants of the two G domain species. The mutation does not induce any significant change of the rates of association or dissociation of these complexes.

GTPase Activity. In contrast to the lack of relevant effects on the interaction with GDP or GTP, substitution of His84 by Gly heavily reduces the catalytic activity. In our standard assay conditions in the presence of 15 μM GTP, the turnover GTPase activity is at the limit of the sensitivity of our method (Figure 1). The calculated value, 0.005 mmol·mol^{-1}·s^{-1}, represents approximately 5% of the activity of wild-type G domain.

Since it is known that the intrinsic GTPase activity of EF-Tu and of its G domain is strongly dependent on the concentration of monovalent cations (Ivell et al., 1981; Jensen et al., 1989), the residual GTPase activity of G domain HG84 was also measured as a function of the concentration of KCl. As illustrated in Figure 2, the activity increases with increasing concentrations of KCl, showing, however, a somewhat different pattern from EF-Tu and G domain wild type. With G domain HG84, a diphasic stimulatory effect becomes evident, since concentrations between 50 and 600 mM KCl do not affect the GTPase activity. At no concentration, however, does the residual activity of the mutant exceed 5% of the activity of wild-type G domain.

The mutation has also no apparent effect on the $MgCl_2$ concentration dependence of the GTPase activity (not shown) nor does it affect the pH dependence of the GTP hydrolysis since this activity, though greatly reduced, displays a broad

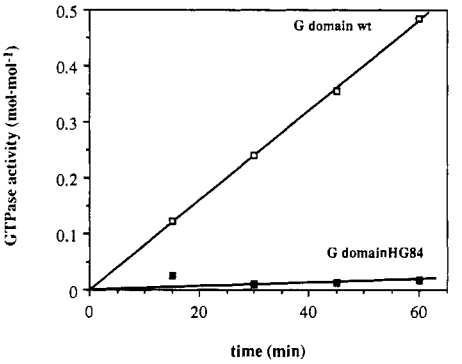

FIGURE 1: GTPase activity of G domain HG84 as compared to G domain wild type. 1.2 μM G domain (□) or G domain HG84 (■) was incubated with 15 μM [γ-^{32}P]GTP (2000 cpm/pmol) in 150 μL of standard buffer (50 mM imidazole acetate, pH 7.7, 50 mM NH_4Cl, 10 mM $MgCl_2$ and 1 mM dithiothreitol) at 30 °C. Samples were taken at the indicated time intervals and analyzed for liberated P_i by the isopropyl acetate method (Parmeggiani & Sander, 1981).

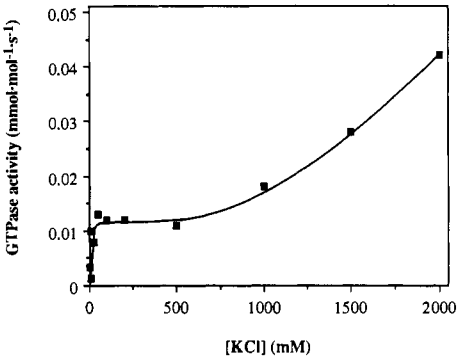

FIGURE 2: GTPase activity of G domain HG84: dependence on the KCl concentration. The GTPase activity was measured after a 30-min incubation at 30 °C of 1.5 μM G domain HG84 with 14 μM [γ-^{32}P]GTP (5200 cpm/pmol) in 30 μL of standard buffer containing the indicated concentrations of KCl as monovalent cations. Aliquots of 25 μL were taken and analyzed for liberated P_i by the charcoal method (Crêchet & Parmeggiani, 1986).

Table II: Effect of Substitution of His84 by Gly on GTPase Activity of the G Domain[a]

	G domain			G domain HG84			
buffer[b]	k_{cat} ($s^{-1} \times 10^3$)	K_m (μM)	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)	k_{cat} ($s^{-1} \times 10^3$)	k_m (μM)	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)	$\Delta(\Delta G^{\ddagger})$[c] ($kJ \cdot mol^{-1}$)
0.05 M NH_4Cl	0.125[d]	8.0[d]	16	nd[e]	nd		
2 M KCl	1.4	7.8	179	0.078	7.2	11	+7.1

[a] In 140 μL of buffer, 1.0–1.5 μM protein was incubated at 30 °C with different concentrations of [γ-^{32}P]GTP (2–60 μM, with specific activity 2750 cpm/pmol). Samples of 40 μL, taken after 20, 40, and 60 min, were analyzed for liberated [^{32}P]P_i by the charcoal method (Crêchet & Parmeggiani, 1986). From these points, the GTPase activity was calculated as moles of hydrolyzed GTP per mole of protein per second. The acquired data were used in Lineweaver–Burk or Eadie–Hofstee plots in order to obtain the K_m and k_{cat} values. [b] Buffer containing 50 mM imidazole acetate, pH 7.6, 10 mM $MgCl_2$, 1 mM dithiothreitol, and monovalent salts as indicated. [c] $\Delta(\Delta G^{\ddagger})$ calculated according to $\Delta(\Delta G^{\ddagger}) = -RT \ln ([k_{cat}/K_m)_{mutant}/(k_{cat}/K_m)_{wild\ type}]$, where R = 8.3143 $J \cdot K^{-1} \cdot mol^{-1}$ and T = 303 K. [d] Taken from Jensen et al. (1989). [e] nd, not determined.

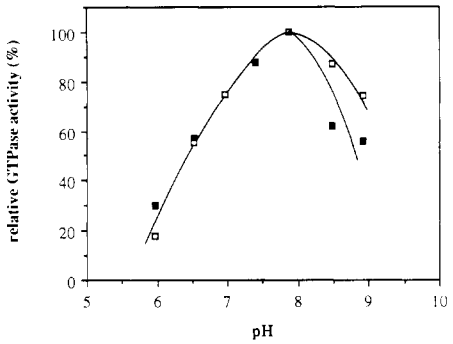

FIGURE 3: pH dependence of the GTPase activity of G domain HG84 compared to wild-type G domain. The reaction mixture of 90 μL containing 50 mM imidazole acetate of the indicated pH, 1 mM $MgCl_2$, 1 M KCl, 12 μM [γ-^{32}P]GTP (4700 cpm/pmol), and 0.6 μM G domain (□) or 1.6 μM G domain HG84 (■) was incubated for 1 h at 30 °C, whereafter the samples were analyzed for liberated P_i with the isopropyl acetate method. The calculated GTPase activities are expressed relative to the maximal activity found.

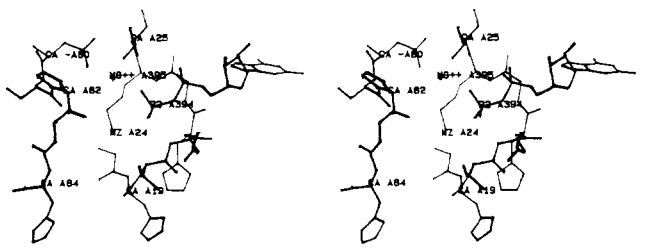

FIGURE 4: Stereodiagram of the structural model of the guanine nucleotide binding pocket of elongation factor Tu in complex with GDP, showing the environment of the phosphate groups of GDP.

optimum around pH 8, like wild-type G domain, as illustrated in Figure 3. The GTPase activities of both G domains have been plotted relative to the maximal activity found in the tested pH range, in order to facilitate the comparison.

Stimulation by monovalent cations of the G domain HG84-dependent GTPase activity allowed us to examine the characteristics of the reaction in more detail. Table II illustrates the different parameters determined for wild-type and mutated G domain. Because of the very low activity, attempts to characterize the reaction at low salt concentrations were not satisfactory in the case of G domain HG84. Concerning wild-type G domain, different ionic conditions in the range of 0.1–2 M KCl induce very small changes of the Michaelis–Menten constant (7–8 μM), within experimental error (not shown). The K_m value of 7.2 μM found for G domain HG84 at 2 M KCl is within this range (Table II). This situation strongly contrasts to the dramatic effect of the substitution on the k_{cat}, which is reduced by a factor of 20. When these data were used to calculate the change in the free energy of activation resulting from the mutation, a value of +7.1 kJ·(mol of protein)$^{-1}$ was obtained (Table II). It is worth mentioning that already in the kirromycin-dependent system it was shown that the apparent Michaelis–Menten constant of the EF-Tu GTPase is little changed as a function of the concentrations of monovalent and divalent cations, in contrast to the large effect on the catalytic activity (Ivell et al., 1981).

Discussion

The His84 of EF-Tu is situated at the edge of the second consensus element (*D80*CPGH84) of the GTP binding proteins and is a conserved residues in elongation and initiation factors from different organisms (Dever et al., 1987). In the other families of GTP binding proteins, most GTPases involved in information signals contain a Gln in the position corresponding to 84 (Dever et al., 1987), though some exceptions are known (Ahnn et al., 1986; Chardin, 1988). In the three-dimensional model of the nicked EF-Tu·GDP complex, the side chain of His84 points away from the phosphoryl groups (la Cour et al., 1985; Jurnak, 1985), as illustrated in Figure 4. The distance of Nδ1 of His84 to the closest oxygen of the β-phosphate is 11.4 Å (J. Nyborg, personal communication). Although this orientation does not allow an interaction between the side chain of His84 and the substrate, neither directly nor via a water molecule, several observations indicate that upon binding of GTP the local conformation of EF-Tu undergoes changes (Kaziro, 1978), perhaps involving a displacement of loop 82–83 (la Cour et al., 1985; Nyborg & la Cour, 1989). This could allow an interaction between His84 and the γ-phosphate. Earlier NMR studies with EF-Tu from *Thermus thermophilus* indicate that a histidine residue is involved in the binding of GTP but not GDP (Nakano et al., 1979).

From our results, substitution of His84 by Gly only induces small, if any, changes of the parameters defining the binding of both guanine nucleotides, as shown by dissociation constants, dissociation rate constants, and stability of the GDP complex to thermal denaturation. This is further supported by the near-identical K_m values of the GTPase activities of mutant and wild-type G domain at 2 M KCl. Thus, His84 does not participate in the binding of the γ-phosphate of GTP.

Since so far no crystal-derived structure of the G domain has been determined, we ought to refer to the structure of the N-terminal domain from the EF-Tu·GDP model which shows that the phosphate groups of GDP are interacting with several main-chain amides of residues belonging to the first consensus sequence element (*G18*HVDH*GKT*25), the side chain of Lys24, the dipole moment of α-helix 24–40, and the bound Mg^{2+} ion (Nyborg & la Cour, 1989; Jurnak et al., 1989). A strong hydrogen bond between a β-phosphate oxygen and the amide of Lys16 or -24 has been recently demonstrated by NMR methods for p21 and G domain, respectively (Redfield & Papastavros, 1990). Side chains of residues in the second consensus element, *D80*CP*G83*, do not seem to participate in the binding of GDP, in line with our observations that the affinities for GDP and GTP are not affected by the mutations His84 → Gly and Pro82 → Thr. However, the latter mutation

shows that, during GTP hydrolysis, the side chain of Thr82 is close enough to the γ-phosphate to be phosphorylated (Cool et al., 1990).

Concerning the molecular mechanism of GTP hydrolysis, Ecclestone and Webb (1983), using a chiral analogue of GTP bound to EF-Tu in complex with the antibiotic kirromycin, have shown that the γ-phosphate undergoes inversion during hydrolysis and that no phosphorylated intermediate could be detected. They interpreted this result as nucleophilic attack of the γ-phosphate by a water molecule. Similar studies revealed the same stereochemistry for the GTPase activity of elongation factor G stimulated by ribosomes (Webb & Ecclestone, 1982) and of p21 (Feuerstein et al., 1989). Swart (1987) proposed an activation of the water molecule by the transfer of a proton via His84 to Glu117, the resulting hydroxyl ion acting as a nucleophile. It is noteworthy that also Glu117 is a conserved residue in all translation factors.

The 20-fold reduction of the GTPase activity is in line with the results obtained with the corresponding mutations of p21Gln61 (Der et al., 1986), RAS1Gln68 (Temeles et al., 1985), and $G_{s,\alpha}$Gln227 (Graziano & Gilman, 1989; Landis et al., 1989), and points to the involvement of His84 in the catalytic mechanism. Substitution of His84 by Gly disrupts (an) interaction(s) in the transition state corresponding to 7.1 kJ·mol^{-1}. This effect is likely to be caused by the removal of one uncharged hydrogen bond, whose binding energy lies in the range of 2.1–7.5 vs 12.6–25.1 kJ·mol^{-1} for a charged hydrogen bond (Fersht et al., 1985; Fersht, 1988). Therefore, the change does not concern a direct interaction with a negatively charged oxygen of the phosphoryl group but is probably mediated by a water molecule. Although the involvement of other residues cannot be excluded, a direct interaction between His84 and a water molecule can be considered as probable. The side chain of His84 either could activate a water molecule, which as a nucleophile would attack the γ-phosphorus of GTP or, alternatively, could interact via a water molecule with an oxygen of the γ-phosphate in the transition state of the GTPase reaction.

The first possibility is supported by the mechanism recently proposed for the GTP hydrolysis of *ras* protein p21. In this protein, it appears that residues 60–67 are very flexible (Pai et al., 1989; Milburn et al., 1990). From the X-ray analysis at high resolution of p21 in complex with the slowly hydrolyzing GppNp, Pai et al. (1990) could distinguish different conformations, one of which places the side chain of Gln61 in hydrogen bond distance to a water molecule properly positioned to carry out the nucleophilic attack. This observation favors a general base catalysis by Gln61 in the GTPase reaction. In extrapolating this crystallographic interpretation to the situation in EF-Tu, one should, however, keep in mind that in contrast to the long, flexible loop 59–65 in p21, the EF-Tu·GDP model shows a very short loop formed by residues 82–83 (Jurnak, 1985; Nyborg & la Cour, 1989), a structural difference recently underlined by Jurnak et al. (1990). It is also worth mentioning that concerning the proton-transfer mechanism, as proposed by Swart (1987), substitution Glu117 → Gln has been found not to affect the GTPase activity of the G domain, ruling Glu117 out as a final acceptor (Harmark et al., 1990). At the present resolution of the crystallographic structure of EF-Tu, other catalytic triads cannot be readily proposed.

The second possible role of His84 in the GTPase reaction is the binding to the transition state via a water molecule. Stabilization by the enzyme of the transition state for the catalyzed reaction is one of the basic features of enzymatic catalysis, as recently reviewed by Kraut (1988). In view of the binding of the guanine nucleotides by multiple interactions with EF-Tu (Nyborg & la Cour, 1989), it is likely that the transition-state configuration of the substrate is to a great extent stabilized by the enzyme, using the binding energies of the interacting residues to lower the energy barrier of the hydrolysis. It is possible that this mechanism constitutes the major catalytic factor, as has been described for the hydrolysis of ATP by tyrosyl- and other aminoacyl-tRNA synthetases (Leatherborrow et al., 1985; Borgford et al., 1987; Fersht et al., 1988) and antibody catalysis (Pollack & Schultz, 1987). In EF-Tu, there are a number of interactions that may favor the catalysis in a more direct way. The γ-phosphate is probably polarized by the complexed Mg^{2+} ion and by the side-chain group of Lys24, in view of the observations that the corresponding Lys16 interacts with the γ-phosphate in the p21·GTP complex (Pai et al., 1989; Milburn et al., 1990). Substitution of Arg201 in the α subunit of G_S, corresponding to Arg58 in EF-Tu (Masters et al., 1986), has been found to evoke a drastic decrease of the GTPase activity (Bourne et al., 1989) and could therefore also be involved in the polarization of the γ-phosphate as has been suggested for the corresponding Thr35 in p21 (Pai et al., 1989). In EF-Tu, however, it is not very likely that the side chain of Arg58 is involved in the GTPase mechanism, since trypsinized EF-Tu, lacking residues 44–58, shows a quite normal polyphenylalanine synthesis activity (Wittinghofer et al., 1980).

The observation that the residual GTPase activity of G domain HG84 is significant and exposed to virtually the same constraints as the activity of wild-type G domain (see cation and pH effect) shows that GTP hydrolysis, though greatly reduced, can still occur in the mutated protein. This implies that His84 is not really essential for the catalysis and suggests a structural role in the GTPase reaction. A similar conclusion was reached by Der et al. (1986) for Gln61 in p21, in view of the uniform 90% reduction in GTPase activity when substituting Gln by 17 different residues. In particular, mutation of Gln61 into His has been shown to reduce the catalytic activity to 10–20% (Der et al., 1986; John et al., 1988). At the same time, the typical dependencies of the GTPase activity of the G domain and EF-Tu on cation concentration and pH can reflect effects on the overall conformation of the protein and not specifically on the catalytic reaction. If so, His84 could exert a catalytic function by activation of a water molecule for nucleophilic attack through base catalysis, but substitution by Gly would not prevent that some hydrolysis of GTP takes place, since the other prerequisites for an active conformation are still present.

In conclusion, His84 is not involved in the binding of the substrate but does take part in the GTPase reaction. Further work will be required to determine uniquivocally whether His84 is a catalytic or a contact residue.

Acknowledgments

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Registry No. GTPase, 9059-32-9; 5′-GTP, 86-01-1; His, 71-00-1.

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