Relevance of histidine-84 in the elongation factor Tu GTPase activity and in poly(Phe) synthesis: its substitution by glutamine and alanine

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Abstract Substitution of His-84 (→Gln and →Ala), a residue of the switch II region of *E. coli* elongation factor (EF) Tu, hardly affected the binding of GTP or GDP. The activity in poly(Phe) synthesis and GTP hydrolysis of EF-Tu H84Q were both reduced to about 35%, as compared to EF-Tu wt, whereas EF-Tu H84A was inactive in poly(Phe) synthesis but still showed a 10% residual GTPase activity. Phe-tRNA^{Phe} exerted a similar inhibitory effect on the GTPase activity of EF-Tu wt and EF-Tu H84Q while abolishing that of EF-Tu H84A. Ribosomes enhanced the GTPase activity of EF-Tu H84Q, but not that of EF-Tu H84A, on which they even seemed to exert an inhibitory effect. The one-round GTP hydrolysis associated with the EF-TuH84Q-dependent binding of Phe-tRNA^{Phe} to poly(U)-programmed ribosomes was less efficient than with EF-Tu wt. Kirromycin stimulated the GTPase activities of both mutants less than EF-Tu wt. The results of this work do not support a catalytic role of His-84 in the intrinsic GTPase of EF-Tu, but they emphasize the importance of its side-chain for polypeptide synthesis and GTP hydrolysis.

Key words: Protein biosynthesis; GTPase; GTP-binding protein; Elongation factor Tu; Site-directed mutagenesis

1. Introduction

Histidine-84 is situated at the edge of the second consensus sequence element (*D*80C*PG*H84) of elongation factor (EF) Tu, that is part of the switch II region. This residue is conserved in elongation factors from different organisms, while in the family of ras proteins the corresponding position is occupied by glutamine [1]. The interest in His-84 rose from the finding that the EF-Tu-dependent GTP hydrolysis proceeds by an in-line attack of a molecule of water [2] and from the 3-D model of human H-ras p21, which suggested that the side-chain of Gln-61 (corresponding to His-84 in EF-Tu) could activate a water molecule found conveniently placed for a nucleophilic attack on the γ-phosphate of GTP [3,4]. However, substitution H84G in the isolated GTP-binding domain of EF-Tu revealed a residual GTPase activity, making it unlikely that H84 is solely responsible for the catalysis [5].

In order to shed further light on its function, His-84 has been substituted by glutamine or alanine in the intact EF-Tu. The former substitution introduces the same residue as found in the homologous position in p21, which shares several properties with histidine including the ability of forming a hydrogen bond; the latter one investigates the effects due to the elimination of interactions but the hydrophobic ones. We did not introduce a glycine, as in the case of the G domain H84G, to avoid possible side-effects associated with the increased backbone flexibility of this residue.

The results show that the substitution H84Q is compatible with polypeptide synthesis and GTPase activity, whereas EF-Tu H84A can only sustain a low intrinsic GTPase activity. This emphasizes the importance of the nature of the side-chain in position 84 for a physiological function of EF-Tu in protein biosynthesis and the associated hydrolysis of GTP.

2. Materials and methods

The substitution of His-84 was performed by using 'unique-site elimination' mutagenesis [6] on the *tufA* gene of EF-Tu *E. coli*, cloned into a pGEX2T vector [7] in-frame with the gene encoding GST [6]. This method utilizes two primers, one to introduce the mutation, the other to eliminate one non-essential restriction site in the EF-Tu gene. The mutagenesis was essentially carried out by using the 'kit' and instructions of Pharmacia. The application of this method to the *tufA* gene will be described in detail elsewhere (I.M. Krab and A. Parmeggiani, in preparation). The overexpression of the GST-fused EF-Tu was induced with IPTG (0.3 mM) at a cell density of 0.6 $Units_{600\ nm}$. Preparation of the cell extracts was carried out by sonication at 0°C. EF-Tu fused with GST could be rapidly purified by affinity chromatography on glutathione-sepharose [7], using 3.5 ml gel per cell extract from 1 liter culture. After washing with 50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 30 mM NH_4Cl and 7 mM ME until no protein was released from the column, the cleavage of the fused GST-EF-Tu was obtained by passing 1 μg/ml thrombin (Sigma) in the same buffer plus 2.5 mM $CaCl_2$ at a flow of 0.3–0.5 ml/min at 4°C. The cleavage leaves EF-Tu with an extra glycine at the N-terminal end, that did not affect the action of EF-Tu (P. Anborgh, unpublished data). EF-Tu wt was also expressed as a GST-fusion and purified like the mutated factors. After thrombin-cleavage EF-Tu was chromatographed on a 5 ml Q-Sepharose column (Pharmacia). A wash with 50 ml of 180 mM KCl in 50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$ and 5 mM DTT containing 200 μM GDP to remove traces of EF-Ts, was followed by a linear KCl gradient (180–350 mM, 30 ml) in the same buffer containing 50 μM GDP and 1 mM DTT.

Poly(U)-directed poly(Phe) synthesis was followed kinetically by determining the amount of material insoluble in hot TCA [8] and the GTPase activity by the liberation of $^{32}P_i$ using the charcoal method [9]. The NH_4Cl-washed 70 S ribosomes were prepared as described [10]. Protein concentration was determined by the method of Bradford [11], using bovine serum albumin as a standard. For further technical details see legends to figures.

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Abbreviations: EF, elongation factor; ME, 2-mercaptoethanol; DTT, dithiothreitol; GST, glutathione-*S*-transferase; GMPPNP, guanosine 5′-[β,γ-imido]triphosphate; PEP, phospho*enol*pyruvate; PK, pyruvate kinase; IPTG, isopropyl-ß-D-thiogalactopyranoside; P_i, inorganic phosphate; TCA, trichloroacetic acid.

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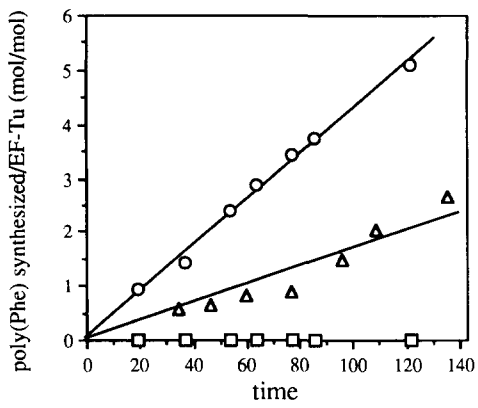

Fig. 1. Activities of EF-Tu wt (○), H84Q (△) and H84A (□) in poly(Phe) synthesis. Final reaction mixture (90 μl) contained: 50 mM Tris-HCl, pH 7.7, 7 mM $MgCl_2$, 80 mM NH_4Cl, 5 mM ME, 30 pmol of EF-Tu wt or mutant, 1 mM PEP, 1.2 μg PK, 0.7 mM GTP, 1.5 mM ATP, 20 pmol EF-Ts, 50 pmol EF-G, 480 pmol [^{3}H]Phe (specific activity 433 cpm/pmol), 450 pmol $tRNA^{Phe}$, Phe-$tRNA^{Phe}$ synthetase, 50 pmol ribosomes with *N*-Ac-Phe-$tRNA^{Phe}$ preloaded P-site and 7 μg poly(U). The reaction was started by adding EF-Tu·GTP. Samples (15 μl) were withdrawn at the indicated times and spotted on Whatman GF/A filters. The phenylalanine incorporation was measured by determining the radioactivity in the hot TCA-precipitate. EF-Tu·GTP was prepared by preincubation (15 min at 30°C) with 3 mM PEP and 3.6 μg PK. Phe-$tRNA^{Phe}$ was obtained by preincubating (30°C for 30 min) 450 pmol $tRNA^{Phe}$ (from a tRNA mixture containing 85% $tRNA^{Phe}$), 480 pmol [^{3}H]Phe (specific activity as above) and saturating amounts of Phe-$tRNA^{Phe}$ synthetase in 20 mM Tris-HCl, pH 7.8, 16 mM NH_4Cl, 8 mM $MgCl_2$, 2 mM ATP and 2 mM DTT.

3. Results

3.1. Purification and general properties of EF-Tu H84Q and H84A

The two mutated factors EF-Tu H84Q and H84A were expressed in *E. coli* almost as efficiently as EF-Tu wt; no anomalies in the cell growth rate were observed. The proteins were overproduced to about 20% of the total proteins of the cell extract. At least 50% of the mutated products remained soluble in the cell extract supernatant after a centrifugation for 15 min at 13,200 × *g*. The yield of the purified products was 8–10 mg per liter of culture. Their activities were stable for at least 1–2 months when kept at −20°C in 50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 60 mM NH_4Cl, 10 μM GDP, 7 mM ME and 50% glycerol.

3.2. Effect of substitutions H84Q and H84A on poly(Phe) synthesis

As shown in Fig. 1, in a poly(Phe) synthesizing system, in which the ribosomal A-site was preloaded with *N*-Ac-Phe-$tRNA^{Phe}$, the activity of EF-Tu H84Q was reduced to about one-third that of EF-Tu wt. EF-Tu H84A was unable to sustain any phenylalanine incorporation.

3.3. Interaction with GDP and GTP

As observed in the case of the G domain H84G [5], the parameters governing the interaction between EF-Tu and GDP or GTP, especially the K_d-values, were not substantially changed in both EF-Tu H84Q and EF-Tu H84A, as compared to EF-Tu wt (Table 1).

3.4. GTPase activity

EF-Tu is a GTPase with a very low intrinsic activity. The antibiotic kirromycin enhances this activity [12]. As shown in Fig. 2A, the GTPase activities of the two mutated EF-Tu's were decreased in comparison to that of EF-Tu wt. The activity of EF-Tu H84Q was around 35% that of EF-Tu wt, whereas the GTPase of EF-Tu H84A was only 10%. Kirromycin had more influence on the GTPase of EF-Tu wt than on that of EF-Tu H84Q and EF-Tu H84A. Its effect was, in the chosen conditions, to stimulate the GTPase activity of EF-Tu wt by almost 8 times, and that of EF-TuH84Q and EF-TuH84A by about 3 times (Fig. 2B).

Phe-$tRNA^{Phe}$ reduced the GTPase activity of EF-Tu wt and of EF-TuH84Q by ~50%, while no activity could be detected with EF-Tu H84A (Fig. 3A). The inhibition of the intrinsic GTPase activity of EF-Tu upon binding of aa-tRNA is a well-known effect [13,14] and likely to be physiologically important for conserving the integrity of the carrier of aa-tRNA to the ribosome. Kirromycin reversed the inhibitory effect of aa-tRNA on the intrinsic GTPase of EF-Tu into a marked stimulation; 30–40 times in the case of EF-Tu wt vs. only 10 times in the case of EF-Tu H84Q (Fig. 3B), whereas no activity at all was observed with EF-Tu H84A. In an assay measuring the

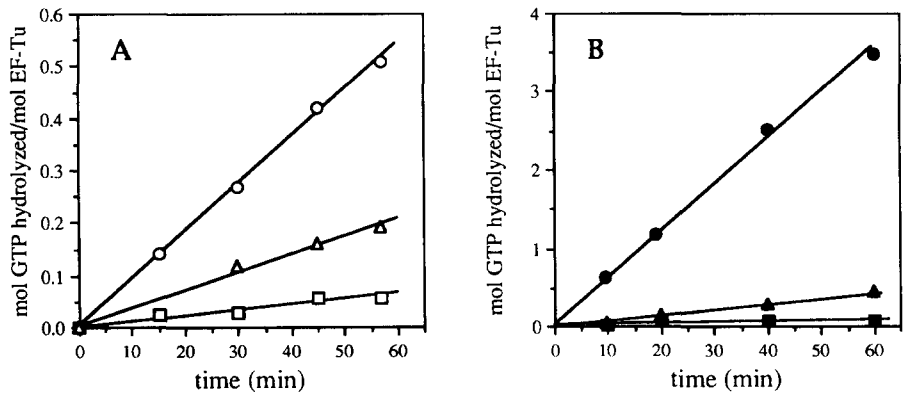

Fig. 2. The intrinsic GTPase activity of EF-Tu wt (○,●), H84Q (△,▲) or H84A (□,■) in the absence (A, open symbols) and the presence of kirromycin (B, filled symbols). (A) Final reaction mixture (90 μl): 50 mM Tris-HCl, pH 7.7, 10 mM $MgCl_2$, 100 mM NH_4Cl, 1 mM DTT, 108 pmol EF-Tu wt or mutant, 1350 pmol [γ^{32}-P]GTP (specific activity 1700 cpm/pmol). The reaction was started by the addition of EF-Tu·[γ^{32}-P]GTP (prepared as described in the legend to Fig. 1) at 30°C. Samples (20 μl) were withdrawn at the given times and the $^{32}P_i$ liberated was measured. (B) With kirromycin (50 μM) the amount of EF-Tu was 72 pmol and of [γ^{32}-P]GTP (specific activity 1280 cpm/pmol) 9180 pmol.

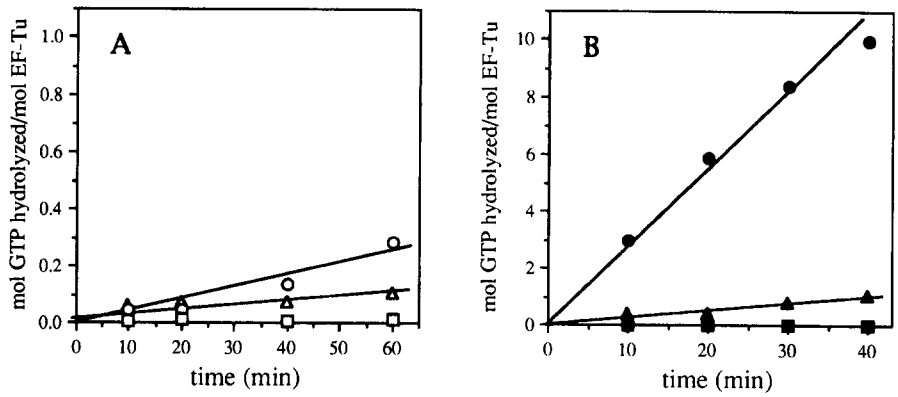

Fig. 3. Effect of Phe-tRNA^{Phe} on the GTPase activity of EF-Tu wt (○,●), H84Q (△,▲) or H84A (□,■) without (A, open symbols) and with kirromycin (B, filled symbols). (A) Final reaction mixture (90 μl): 72 pmol of either wild-type or mutated EF-Tu, 144 pmol Phe-tRNA^{Phe}, 1400 pmol [γ^{32}-P]GTP (specific activity 1252 cpm/pmol) in the same buffer as in Fig. 2. The reaction was started by the addition of EF-Tu·[γ^{32}-P]GTP (see legend to Fig. 1) at 30°C. tRNA^{Phe} was aminoacylated as reported in the legend to Fig. 1, except that 500 pmol tRNA^{Phe} (from a tRNA mixture containing 1.8% tRNA^{Phe}) and 1000 pmol Phe were used. At the given times, samples (20 μl) were withdrawn and the $^{32}P_i$ liberated measured. (B) With kirromycin (50 μM), the amount of [γ^{32}-P]GTP was 1700 pmol (specific activity 1500 cpm/pmol).

kinetics of the spontaneous deacylation of Phe-tRNA^{Phe} under similar experimental conditions, EF-Tu H84A stabilized the ester bond of the aa-tRNA with efficiency indistinguishable from that of EF-Tu wt, whereas EF-Tu H84Q was slightly less efficient (data not shown).

The results obtained with the addition of non-programmed ribosomes to the preformed EF-Tu·[γ^{32}-P]GTP complex are illustrated in Fig. 4A. The GTPase of EF-Tu wt showed an initial fast phase, corresponding to 0.8–0.9 mol GTP hydrolyzed per mol EF-Tu [13]. The ribosomes also enhanced the GTPase activity of EF-Tu H84Q but in this case the initial burst was either not present or much reduced (the latter effect not illustrated). In contrast, the turnover GTPase of EF-Tu H84Q displayed the same rate as EF-Tu wt. These results show that the stimulation of the catalytic activity of EF-Tu H84Q·GTP by the ribosome is less efficient than with EF-Tu wt, as shown by the deficient initial phase. Unlike this, the rate of the turnover GTPase of EF-Tu wt and EF-Tu H84Q is similar, since the rate-limiting step is now the regeneration of the EF-Tu·GTP complex rather than the GTP hydrolysis [13] and the interaction with GTP and GDP is hardly changed by mutation H84Q (Table 1). Kirromycin eliminated the difference between initial and turnover GTPase activity of EF-Tu wt·GTP (Fig. 4B), the activity corresponding to the initial velocity in the absence of the antibiotic (Fig. 4A and B). This effect is a consequence of the property of kirromycin to change the rate-limiting step in the EF-Tu GTPase, which is no longer the regeneration of the EF-Tu·GTP complex but the breakdown of GTP, due to the strong stimulation of the GDP/GTP exchange by the antibiotic [15]. Ribosomes did not enhance the GTPase activity of EF-Tu H84A, on which they even appeared to exert an inhibitory effect. Kirromycin did not change this picture.

With poly(U)-programmed ribosomes plus Phe-tRNA^{Phe}, i.e. under conditions leading to the EF-Tu·GTP-mediated binding of Phe-tRNA^{Phe} to the ribosomal A-site, EF-Tu wt displayed a burst-like hydrolysis of GTP, corresponding to a 1-to-1 stoichiometric ratio with respect to the molar amount of EF-Tu (Fig. 5). Also EF-Tu H84Q showed an initial burst-like activity that was, however, only one-third that of EF-Tu wt, after which the extent of GTP hydrolyzed steadily increased to a level close to that of EF-Tu wt. In experiments not illustrated, the kinetics of binding of Phe-tRNA^{Phe} to the poly(U)·ribosome mediated by EF-Tu H84Q gave the same results as EF-Tu wt.

4. Discussion

The high resolution 3-D model of the complex of EF-Tu *Thermus thermophilus* with GMPPNP shows that in EF-Tu there is a water molecule that could act as nucleophile on the γ-phosphate of GTP [16]. However, the existence of a 'hydrophobic gate', formed by Val-21 and Ile-61 (Val-20 and Ile-60, respectively, in *E. coli* EF-Tu), situated between His-85 (His-84 in *E. coli*) and this water molecule makes an activation of the water molecule by the side-chain of His-85 improbable at least for the slow intrinsic GTPase activity of EF-Tu. Differently from EF-Tu, in one of the conformations of the flexible switch II region of p21, the side-chain of Gln-61 points to a water molecule in line with the γ-phosphate of GTP, without the interference of a hydrophobic barrier [3]. In p21, the possibility that Gln-61 activates the nucleophilic water molecule was questioned [17,18]. Langen et al. [18] suggested, on the basis of theoretical considerations, that the γ-phosphate itself could act

Table 1
Effect of the substitution of His-84 by Gln or Ala on the GDP or GTP interaction of EF-Tu

System	K_d (nM)	$10^4 k_{-1}$ (s^{-1})	k_{+1} ($s^{-1} \cdot nM^{-1}$)
EF-Tu wt·GDP	4.5	3.0	90
EF-Tu H84Q·GDP	3.8	6.8	180
EF-Tu H84A·GDP	2.8	8.0	290
EF-Tu wt·GTP	640	130	20
EF-Tu H84Q·GTP	580	200	50
EF-Tu H84A·GTP	510[a]	220	40[a]

The various parameters were measured at 0°C, as reported (Cool and Parmeggiani [5]; Anborgh and Parmeggiani, 1993). The K_d was derived from the ratio k_{-1}/k_{+1}.
[a] In the case of EF-Tu H84A, due to difficulties in measuring the k_{+1}, the K_d was determined directly by nitrocellulose binding assay using concentrations of [^{3}H]GTP (specific activity 1437 cpm/pmol) from 0.115 to 7.0 μM; the K_d-value was then calculated via a Scatchard plot [23].

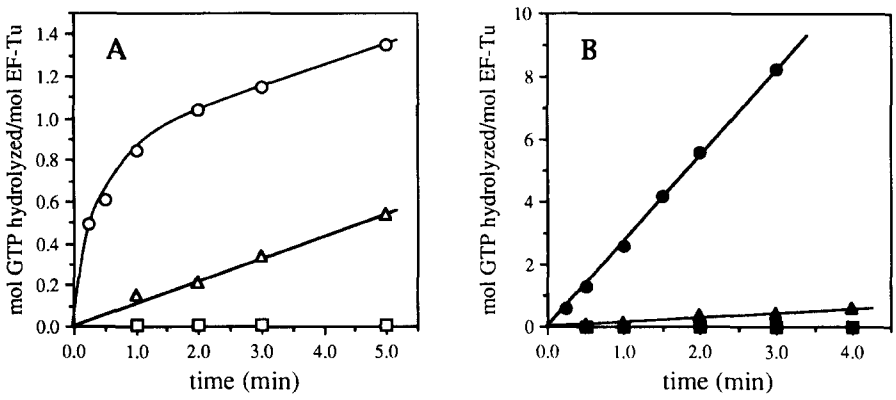

Fig. 4. The GTPase activity of EF-Tu wt (○●), H84Q (△,▲) or H84A (□,■) in the presence of ribosomes without (A, open symbols) and with kirromycin (B, filled symbols). (A) Final reaction mixture (120 μl): 96 pmol of EF-Tu wt or mutant, 192 pmol ribosomes, 1200 pmol of [γ^{32}-P]GTP (specific activity 560 cpm/pmol) in 50 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 100 mM KCl and 7 mM ME. The reaction was started at 30°C by the addition of ribosomes. The liberated $^{32}P_i$ was determined on 20 μl samples. EF-Tu·[γ^{32}-P]GTP was prepared as reported in the legend to Fig. 1. (B) With kirromycin (50 μM), the amount of [γ^{32}-P]GTP used for the EF-Tu reaction mixture was 3000 pmol with a specific activity of 460 cpm/pmol.

as a proton acceptor and that the role of Gln-61 would be only to stabilize the nucleophile by a hydrogen bond. Very recently experimental evidence has been described supporting this model [19], that could also be in part valid for the intrinsic GTPase of EF-Tu [20]. However, a different situation is likely to underlie the ribosomal EF-Tu-dependent GTP hydrolysis. The interaction of EF-Tu·GTP with mRNA-programmed ribosomes during the binding of aa-tRNA can increase the velocity of the GTPase reaction by 5 orders of magnitude, as derived from experiments reported in [13,21]. In these conditions, the need for an activation mechanism is evident.

A number of conclusions can be drawn from the results of this work. Whereas the binding of GTP or GDP is little or not affected by substitutions H84A and H84Q, the GTPase activity of EF-Tu is modified under all conditions tested. This emphasizes the relevant role of H84 in this activity. The presence of a protonated side-chain of the 84 residue, though important, does not appear to be essential for the intrinsic GTPase of EF-Tu, as shown by the low but significant hydrolysis sustained by EF-Tu H84A. In this regard, the action of alanine resembles that of glycine in the isolated EF-Tu GTP-binding domain carrying the substitution H84G [5]. The residual GTPase activity observed with EF-Tu H84A is compatible with the recent model for the p21 intrinsic GTPase (see above and [18]). However, for an efficient function of EF-Tu in the process of elongation, the properties of the side-chain of histidine become essential. Glutamine shares only in part the properties of histidine, as shown by the lower activity of EF-Tu H84Q in poly(Phe) synthesis and GTPase, whereas alanine is unable to substitute for histidine in the physiological functions. Whether the specific properties of the side-chain of histidine concern its ability to abstract a proton, the resulting positive charge or its capability of forming a hydrogen bond, remains to be clarified.

Concerning the macromolecular ligands of EF-Tu, both the experiments in Figs. 4 and 5 indicate that the ribosome does not activate the EF-Tu H84A GTPase but can induce a catalytically active state of EF-Tu H84Q, though less efficient than that of EF-Tu wt. In the presence of μM concentrations of Phe-$tRNA^{Phe}$, that are largely saturating (the affinity of aa-tRNA for EF-Tu·GTP lies in the nmolar range [22]), the GTPase of both mutants is lowered, in EF-Tu H84A practically abolished. In the case of EF-Tu H84Q the slower GTP hydrolysis accompanying this reaction is not due to a defective interaction with aa-tRNA, but rather to a negative influence on the catalytically active state, since this mutant can still effectively support the enzymatic binding of Phe-$tRNA^{Phe}$ as well as protect the ester bond of Phe-$tRNA^{Phe}$ against spontaneous deacylation.

To conclude, in elongation the action of the mRNA-programmed ribosome on EF-Tu·GTP·aa-tRNA may induce such a dramatic conformational change of the factor as to allow the efficient activation of a water molecule as a nucleophile by an as yet unidentified residue that could be represented by His-84. Alternatively, His-84 could perform an important indirect action in GTP hydrolysis, as suggested for the intrinsic

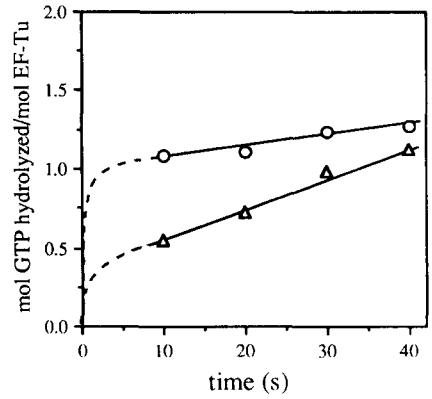

Fig. 5. The GTPase activity of EF-Tu wt (○) and H84Q (△) in the presence of Phe-$tRNA^{Phe}$ and poly(U)-programmed ribosomes. Final reaction mixture (65 μl): 50 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 100 mM KCl, 72 pmol EF-Tu, 346 pmol ribosomes, 346 pmol Phe-$tRNA^{Phe}$, 168 μg poly(U), 918 pmol [γ^{32}-P]GTP (specific activity 1812 cpm/pmol) and 1 mM DTT. EF-Tu·[γ^{32}-P]GTP and Phe-$tRNA^{Phe}$ (from a tRNA mixture containing 1.8% $tRNA^{Phe}$) were prepared as described in the legend to Fig. 1. The reaction was started at 30°C by the addition of poly(U)-programmed ribosomes preloaded with $tRNA^{Phe}$ in the P-site. Samples (20 μl) were withdrawn at the given times and the liberated $^{32}P_i$ was measured. When this assay was performed with 85% pure $tRNA^{Phe}$ or with ribosomes preloaded with *N*-AcPhe-$tRNA^{Phe}$, similar results were obtained.

GTPase [5], by favoring the active state of the GTP binding pocket of EF-Tu.

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