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Divergent effects of fluoroaluminates on the peptide chain elongation factors EF-Tu and EF-G as members of the GTPase superfamily

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Fluoroaluminates are thought to mimic the γ-phosphate of GTP and thus, together with GDP, perturb the functioning of heterotrimeric GTP-binding G-proteins. Here we show they do inhibit the ribosome-stimulated GTPase activity of EF-G from *Escherichia coli* via the formation of a stable complex with EF-G·GDP and ribosomes. In contrast, no perturbed interactions were observed in a similar ribosomal complex with EF-Tu. Interestingly, in the absence of ribosomes both EF-Tu and EF-G remain totally unaffected by fluoroaluminates. For members of the GTPase superfamily such differential effects have not been described before.

Guanine-nucleotide binding centre; γ-Phosphate analogue; GTPase effector; G-protein; Translational inhibition

1. INTRODUCTION

Proteins that bind and hydrolyse GTP perform an unprecedented variety of functions [1–3]. Each of these proteins act as a molecular switch of which the 'on' and 'off' states are triggered by binding and hydrolysis of GTP to GDP, respectively. Fluoride and aluminium ions perturb the functioning of a number of these proteins [4–12]. This is thought to be due to their presence in the γ-phosphate binding site of the guanine-nucleotide binding centre if the latter is occupied by GDP [7–12].

According to the proposed models of perturbation, fluoride and aluminium ions should therefore inhibit both the bacterial peptide-chain elongation factors EF-Tu and EF-G, two representative members of the GTPase superfamily. We previously found, however, that fluoroaluminates do not induce a 'GTP-like' conformation in EF-Tu·GDP [13]. Furthermore, others showed that fluoroaluminates do not bind to EF-Tu at all [14]. Unexpectedly, however, we did find an inhibition by fluoroaluminates of the peptide-chain elongation cycle [13].

In the present research we have investigated whether EF-G, the other GTP-binding elongation factor, might be the target responsible for the observed inhibition by fluoroaluminates.

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2. MATERIALS AND METHODS

2.1. *Chemicals*

$AlCl_3 \cdot 6H_2O$ and NaF were from Merck (Darmstadt, Germany) in the purest grades available; solutions were always kept in plastic vials. Cellulose polyethyleneimine sheets and 2-propanol were from J.T. Baker (Phillipsburg, NJ, USA). [^{3}H]GDP (12.4 Ci/mmol), ([^{3}H]GTP (9 Ci/mmol) and [γ-^{32}P]GTP (2.97 Ci/mmol) were from Amersham International pcl (Amersham, UK). The nitrocellulose sheets were from Schleicher & Schuell (Dassel, Germany).

2.2. *Isolation of elongation factors and ribosomes*

EF-Tu and EF-G were isolated as reported in [15]. EF-G was purified further on a DEAE column using a linear gradient of 0–0.4 M KCl in a sodium cacodylate buffer [16]. To improve purity even more, both elongation factors were rechromatographed on a monoQ column from Pharmacia (Uppsala, Sweden) using a gradient of 0–0.5 M KCl in 'isolation buffer' [15]. In this way, EF-Tu was freed from a trace contamination with EF-G normally present in EF-Tu preparations such as used in [13]. The ribosomes used were washed twice in 1 M NH_4Cl in order to remove elongation factors [17] and stored in 10 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 60 mM NH_4Cl, 10 mM 2-mercaptoethanol, 5% (v/v) glycerol at −80°C.

2.3. *GTPase activities of the elongation factors*

All GTPase activities were studied in 50 mM Tris-HCl pH 7.6, 8 mM $MgCl_2$, 60 mM NH_4Cl, 7 mM 2-mercaptoethanol, 57 μM GTP, 20% (v/v) 2-propanol if indicated, and fluoride and aluminium ions as indicated. In the case of ribosome-stimulated GTPase activities, the reaction mixtures (60 μl) further contained 0.13 μM 70S ribosomes and either 0.025 μM EF-G or 0.025 μM EF-Tu with 0.5 μM kirromycin. After separate preincubation at 37 °C for 10 min of both the elongation factor and the ribosome mixture containing [^{3}H]GTP, they were mixed and the GTPase activity was measured at 37 °C. In experiments on the intrinsic and 2-propanol stimulated GTPase activity of EF-G, the reaction mixtures (60 μl) contained 2.8 μM EF-G.

Each time 12 μl samples were drawn and added to 3 μl 25% (v/v) formic acid in order to stop the GTPase reaction. After centrifugation in an Eppendorf centrifuge, 6 μl portions were spotted on a polyethyleneimine thin-layer plate together with 4 μl of a reference mixture (1 mM GTP, 1 mM GDP) to enable UV detection (254 nm). The plate

was chromatographed in 1 M formic acid, 1 M LiCl. The spots of GTP and GDP were cut out and the ^3H determined by liquid scintillation counting.

2.4. *EF-G·guanine-nucleotide·ribosome·fluoroaluminate complexes*

The formation of stable complexes between EF-G, guanine nucleotide and ribosomes were studied in 50 mM Tris-HCl pH 7.6, 10 mM magnesium acetate, 100 mM potassium glutamate, 0.3 mM K_2HPO_4, 10 mM 2-mercaptoethanol, containing either 7.5 μM [^{3}H]GDP (1574 dpm/pmol) or [^{3}H]GTP plus [γ-^{32}P]GTP (^{3}H: 1104 dpm/pmol; ^{32}P: 2136 dpm/pmol). Where indicated, either 2 mM fusidic acid or 1 mM fluoride together with 30 μM aluminium ions were included. Mixtures of 67 pmol EF-G in 95 μl were preincubated at 37°C for 5 min, after which the ribosomes (73 pmol in 5 μl of storage buffer) were added and the complete mixtures were incubated for another 5 min. Thereafter, the reaction mixtures were cooled down to 0°C, filtered on nitrocellulose membrane filters, washed once with 0.5 ml of cold buffer (50 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$) and the retained radioactive guanine-nucleotides were measured by liquid scintillation counting. The inclusion of 0.3 mM K_2HPO_4 in the reaction mixtures led to a 4-fold decrease of the background level of nucleotides retained.

3. RESULTS

3.1. *Effects on the ribosome-induced GTPase activity of EF-G and EF-Tu·kirromycin*

In our analysis of the effects of fluoroaluminates, the observed inhibition on polyPhe-synthesis could not be explained by any perturbing effects on EF-Tu itself [13]. Another target of the elongation cycle that could be responsible for the observed effect of fluoride and aluminium ions is the elongation factor EF-G·GTP, that catalyses the translocation.

The effects of fluoride and aluminium ions on the EF-G·ribosome GTPase activity are shown in Fig. 1. A strong inhibition can be observed upon addition of fluoride, which becomes even stronger when both salts are present. In the latter case, the level of the remaining GTPase activity even becomes lower than the background level for ribosomes alone without fluoroaluminates. This may point to a small contamination by EF-G of the ribosome preparation used, since ribosomes alone with fluoroaluminates display the same GTPase activity (not illustrated). The relatively high background level is caused by the chosen excess of ribosomes over EF-G in the reaction mixture: even extensively washed ribosomes contain some endogenous GTPase activity not involved in polypeptide synthesis and accompanied by ATPase activity [18]. The addition of $AlCl_3$ alone does not affect the hydrolysis of GTP.

Although our previous study [13] did not show any effect of fluoroaluminates on EF-Tu alone, nor on the binding of Phe-tRNA to ribosome·polyU complexes in the presence of EF-Tu·GDP, the ribosomal interaction with its GTPase centre had not yet been studied. Therefore, EF-Tu was used in similar GTPase experiments as with EF-G. However, the ribosome-induced GTPase activity of EF-Tu, as compared to EF-G, is much lower. In order to obtain comparable GTPase activities of EF-Tu as with EF-G, the antibiotic kirromycin was added

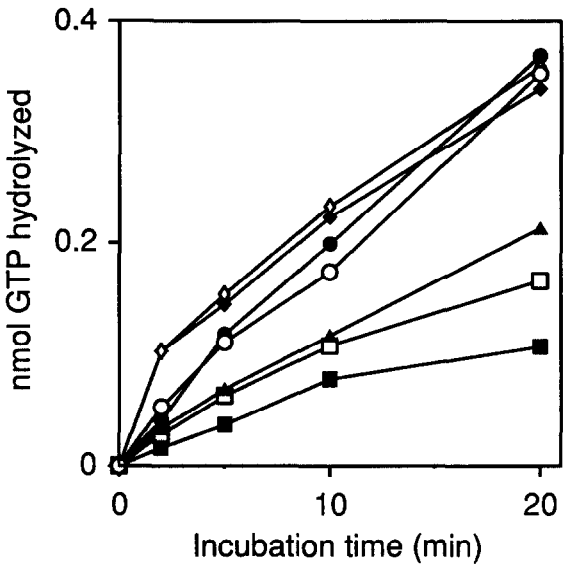

Fig. 1. Effects of fluoride and aluminium ions on the GTPase activity of EF-G and EF-Tu·kirromycin as induced by ribosomes at 37°C. The reaction mixtures with EF-G contained either no further additions (○), 30 μM $AlCl_3$ (●), 1 mM NaF (▲), or 30 μM $AlCl_3$ and 1 mM NaF (■). The reaction mixture with EF-Tu and kirromycin contained either no further additions (◇) or 30 μM $AlCl_3$ and 1 mM NaF (◆). The GTPase activity of the ribosomes alone (□) was used as a reference. For further details see section 2.

to enhance the exchange of GDP for GTP [19]. Interestingly, the ribosome-stimulated EF-Tu·kirromycin GTPase activity is not perturbed upon addition of fluorides (not illustrated) or fluoroaluminates as clearly shown in Fig. 1. The very small EF-Tu GTPase activity induced by ribosomes without kirromycin is not affected either (not illustrated). The results of Fig. 1 thus show that the ribosome-stimulated GTPase activities of the elongation factors EF-Tu and EF-G have a strikingly different response to the presence of fluoroaluminates.

3.2. *Effects on the GTPase activity of EF-G alone, either in absence or presence of 2-propanol*

For further analysis of this difference in response of EF-G we wanted to know whether fluoroaluminates would also affect the guanine-nucleotide binding site on EF-G in the absence of other macromolecular activators. Contrary to possible expectations, Fig. 2 clearly indicates that fluoride and aluminium ions do not inhibit the low intrinsic GTPase activity of EF-G alone, nor the enhanced activity (see [20]) in the presence of 2-propanol. The slight stimulation by fluoroaluminates shown by the latter curve is not very significant.

3.3. *Effects on complex formation between EF-G, guanine nucleotide and ribosome*

The finding that fluoroaluminates specifically interfere in the EF-G·ribosome interaction (Fig. 1) reminded

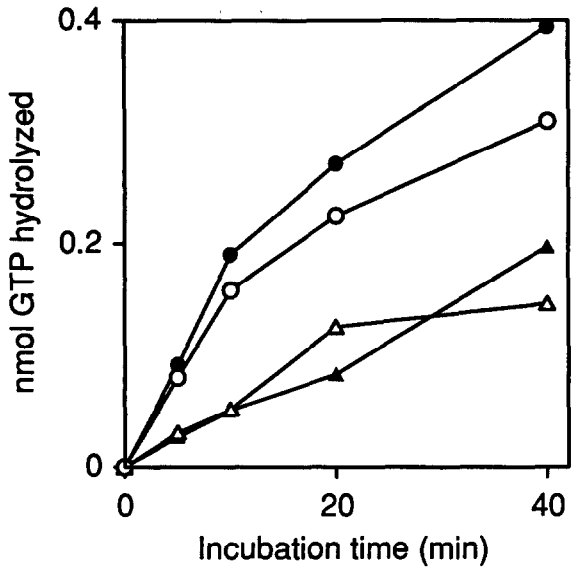

Fig. 2. Effects of fluoride and aluminium ions on the intrinsic GTPase activity of EF-G in the absence or presence of 2-propanol at 37°C. The activities of EF-G with either no further additions (△), 30 μM $AlCl_3$ and 1 mM NaF (▲), 20% (v/v) 2-propanol (○) or 30 μM $AlCl_3$, 1 mM NaF and 20% (v/v) 2-propanol (●) are shown. For further details see section 2.

us to the well-known effect of fusidic acid in such a system [21, 22]. Both agents were therefore compared in their enhancement of GDP and GTP binding affinities by means of the nitrocellulose membrane filtration assay.

In Fig. 3 it can be seen (left panel), that normally the EF-G affinity for GDP and GTP either in the presence or without ribosomes ('standard' or '-ribos.', respectively) is so small that no significant amounts are retained above the background level ('-EF-G'). With fusidic acid (middle panel), however, a strong 1:1 binding of GDP to EF-G·ribosome complexes takes place. Our EF-G·GDP binding value of 40% of the nominal ribosome content compares very well with the data of [21]. In the presence of GTP a first round of GTP hydrolysis is allowed by fusidic acid, but the turnover GTPase reaction is inhibited by the slow dissociation of the GDP formed [22]. This is exactly the case in the middle panel; in the binding experiment with double-isotope labelled GTP only GDP is found back, at the same level as in the preceding GDP binding experiment.

With fluoroaluminates (Fig. 3, right panel) the binding results with GDP and GTP are qualitatively the same as with fusidic acid. For GDP the resulting affinity seems weaker than in the complex with fusidic acid, since in both panels the binding reactions have reached equilibrium values (results not shown). With regard to GTP also fluoroaluminates allow a first round of GTP hydrolysis and the resulting GDP remains firmly bound to the EF-G·ribosome complex (the background level of bound GTP being about the same as in the left panel). In the case of EF-G alone no effect by fluoroaluminates on GDP binding can be found whatsoever.

3.4. *Other effects on the ribosomal complex*

In principle also other interactions of fluoroaluminates with the ribosome might contribute to the inhibition of polyPhe-synthesis as observed in [13], since both the RNA and protein molecules in the ribosomal complex contain potential ligands for those ions. We therefore studied the effect on the peptidyl transferase activity of the ribosome, which is dependent on a correct folding of RNA and proteins but not on GTP binding or hydrolysis. Using the well-known puromycin assay with *N*-acetyl-Phe-tRNA bound at the ribosomal P-site [23] we found no effects of fluoroaluminates on the peptidyl transferase reaction (data not illustrated).

4. DISCUSSION

Fluoroaluminates severely inhibit the GTPase activity of ribosome-bound EF-G involved in the translocation reaction (Fig. 1). The latter result is very similar to the overall effect of fluoroaluminates on polyPhe-synthesis in our previous publication [13]. However, the data of Fig. 2 illustrate that not the intrinsic, but only

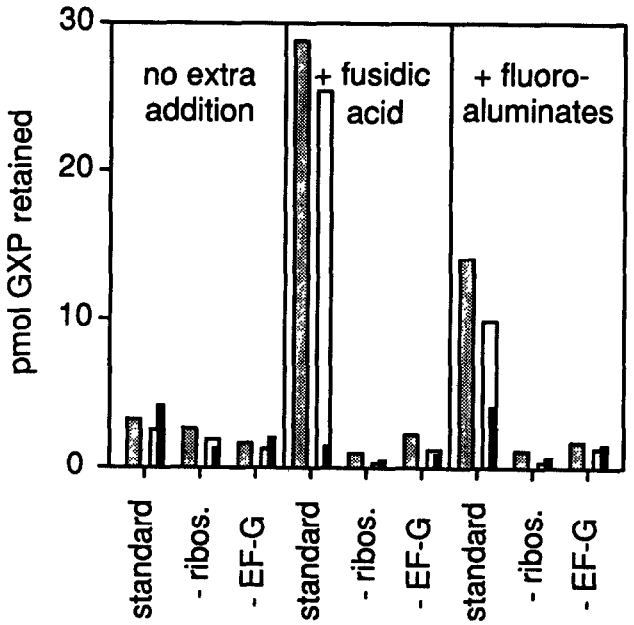

Fig. 3. Effects of fluoroaluminates and fusidic acid on the formation of stable EF-G·GDP complexes with ribosomes, as studied with nitrocellulose membrane filtration. Standard mixtures ('standard') contained EF-G and ribosomes in the presence of GXP: either [^{3}H]GDP (indicated by dotted bars) or GTP with [^{3}H]guanine (open bars) and [γ-^{32}P]phosphate (thin filled bars) were used. In control mixtures either ribosomes ('-ribos.') or EF-G ('-EF-G') were left out. The set of mixtures contained either no further additions (left panel), fusidic acid (middle panel), or fluoroaluminates (right panel). For further details see section 2.

the ribosome-stimulated GTPase activity of EF-G is perturbed. A satisfactory explanation of the GTPase behaviour of EF-G in response to fluoroaluminates in the presence and absence of ribosomes (Figs. 1 and 2, respectively) is given in Fig. 3. The blockage of the ribosome-induced GTPase reaction of EF-G is due to the immobilization of GDP. Without ribosomes no GDP becomes trapped by the fluoroaluminates and, indeed, the intrinsic GTPase of EF-G is unaffected. With respect to GTP affinity and γ-phosphate cleavage no perturbation by fluoroaluminates can be detected. Altogether, the EF-G data could support the γ-phosphate mimicking model for fluoroaluminates [7,12] on the assumption that the associated ribosome somehow contributes to a proper γ-phosphate binding site. However, the striking parallel in the inhibitory action of such different structures as the inorganic fluoroaluminates and the steroidal antibiotic fusidic acid (Fig. 3) makes clear that an indirect and still unknown mechanism cannot be excluded.

For the related GTP-binding domain containing elongation factor EF-Tu the ribosome apparently does *not* contribute to the γ-phosphate binding site. The ribosome-stimulated GTPase reaction is not affected by fluoroaluminates (see Fig. 1 and text), and we previously showed [13] that fluoroaluminates do not interfere with EF-Tu·GDP and Phe-tRNA binding to the ribosome·polyU complex. In retrospect, the inhibition of polyPhe-synthesis [13] was caused by the effect of fluoroaluminates on a catalyzing trace of EF-G in the EF-Tu preparation used (see section 2).

The divergent behaviour of ribosome-bound EF-G and EF-Tu towards fluoroaluminates becomes similar when studied in the absence of the ribosome as the GTPase effector. As mentioned above, fluoroaluminates do not mimic the γ-phosphate in the GTP-binding centre of EF-G alone. For EF-Tu, similar results were obtained by us [13] and others [14]. Moreover, it was recently shown [24] that fluoroaluminates do not perturb the functioning of six smaller (20–25 kDa) GTP-binding proteins either. These findings indicate that the presence of a guanine-nucleotide binding domain with bound GDP is as such not sufficient for a protein to trap fluoroaluminates at the vacant γ-phosphate site.

The latter mechanism was found responsible for the activation of the heterotrimeric G-proteins [4–12], albeit that some findings point to a more complex mechanism [25]. Could the fluoroaluminates sensitivity of the G-proteins perhaps be due to the proximity of an *internal* GTPase effector domain (as postulated by [3]), and thus resemble the case of EF-G with its external effector, the ribosome? If so, new perspectives open up for studying the trigger mechanism in the superfamily of GTPase-switch molecules.

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