Effect of propan-2-ol on enzymic and structural properties of elongation factor G

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Elongation factor G (EF-G) can support a GTPase activity *in vitro* even in the absence of ribosomes when propan-2-ol is present [GTPase^p; De Vendittis, Masullo & Bocchini (1986) *J. Biol. Chem.* **261**, 4445–4450]. In the present work the GTPase^p activity of EF-G was further studied by investigating (i) the effect of ionic environment on GTPase^p and (ii) the influence of propan-2-ol on the molecular structure of EF-G as determined by fluorescence and c.d. measurements. In the presence of 1–300 mM univalent cations (M⁺) alone, no detectable GTPase^p activity was measured; however, in the presence of 1 mM-Mg²⁺ a considerable stimulation was observed at 40 mM-Li⁺ or 75 mM-NH₄⁺. Among bivalent cations (M²⁺), 1 mM-Sr²⁺, 2–5 mM-Ca²⁺ and 1 mM-Ba²⁺ were the most effective, but, in the presence of 75 mM-NH₄⁺, Mg²⁺ and Mn²⁺ became the most efficient, whereas the stimulation by other M²⁺ species was considerably decreased. C.d. measurements showed that the alcohol increased the mean molar residue ellipticity of EF-G at 285 nm, but not at 220 nm. As estimated from fluorescence measurements, in the presence of 20% (v/v) propan-2-ol the value of the dissociation constant of the complex formed between EF-G and 8-anilino-1-naphthalene-sulphonate decreased from 8 to 5 μM; similarly, the number of binding sites on EF-G for the fluorescent probe decreased from 13 to 6. Finally, the alcohol enhanced the quenching of the intrinsic fluorescence of EF-G caused by either acrylamide or KI. The data support the hypothesis that propan-2-ol induces moderate conformational changes of EF-G that make the catalytic centre accessible to the substrate even in the absence of ribosomes. Kinetics of GTPase^p studied at different temperatures did not reveal additional structural changes of EF-G occurring with time or temperature.

INTRODUCTION

In the process of protein synthesis in *Escherichia coli*, the translocation step promoted by EF-G is coupled to the hydrolysis of one molecule of GTP [for reviews see Miller & Weissbach (1977) and Kaziro (1978)]. This EF-G GTPase activity has been investigated in various ribosome-dependent systems, either uncoupled or coupled to polypeptide elongation (Parmeggiani & Sander, 1981).

In a previous paper we reported that EF-G, in an appropriate propan-2-ol/water medium, is able to hydrolyse GTP even in the absence of ribosomal subunits (De Vendittis *et al.*, 1986). Activity of this propan-2-ol-dependent EF-G GTPase, hereafter termed GTPase^p, is about three orders of magnitude lower than that of EF-G GTPase stimulated by ribosomes. This finding clearly showed that EF-G harbours the catalytic site for GTP hydrolysis, thus reproducing the property of the elongation factor Tu, which also possesses a ribosome-independent GTPase activity (Parmeggiani & Sander, 1981); this is also in agreement with the observation that G-proteins possess an intrinsic GTPase activity (Gilman, 1987). Since the GTPase activity of EF-Tu assayed either in the presence or in the absence of ribosomes, as well as in the presence of kirromycin (Parmeggiani & Sander, 1981), is strongly regulated by univalent and bivalent

cations (Ivell *et al.*, 1981), in the present work we tried to find appropriate ionic conditions to enhance the GTPase^p of EF-G and to compare the results with those related to the GTPase activity stimulated by ribosomes (hereafter termed GTPase^r). The effect of other components interacting with EF-G, such as acylated or deacylated tRNA and the antibiotic fusidic acid, an inhibitor of GTPase^r (Bodley *et al.*, 1970), was also investigated, as well as the temperature-dependence of both GTPase^p and GTPase^r. Finally, the relationship between activation of EF-G GTPase by propan-2-ol and structural transitions induced by the alcohol on the factor has been studied by measurements of c.d., of ANS fluorescence of EF-G·ANS and of quenching of the intrinsic fluorescence of EF-G by acrylamide or KI.

MATERIALS AND METHODS

EF-G was obtained from *E. coli* MRE 600 cells and purified by the procedures previously described (Fasano *et al.*, 1978; De Vendittis *et al.*, 1986). This sample of EF-G was at least 99% pure on SDS/polyacrylamide-gel electrophoresis, and was stored at –25 °C in TMAD buffer [20 mM-Tris/HCl (pH 7.8)/1 mM-dithiothreitol/10 mM-MgCl₂/50 mM-NH₄Cl] containing 50% (v/v) glycerol; 1 μg of EF-G was taken to correspond to

Abbreviations used: EF-G, elongation factor G; ANS, 8-anilino-1-naphthalenesulphonate; GTPase^p and GTPase^r, GTPase activity supported by EF-G in the presence of propan-2-ol or ribosomes respectively; M⁺, univalent cation; M²⁺, bivalent cation.

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12.9 pmol (Ovchinnikov *et al.*, 1982). GTP was purchased from Boehringer (Mannheim, Germany); dithiothreitol was from Schwarz/Mann; acrylamide was from Bio-Rad; ANS was from Kodak; fusidic acid was a gift from Sigma Tau (Pomezia, Italy); [γ - 32 P]GTP was from The Radiochemical Centre (Amersham, Bucks., U.K.). All other reagents were of analytical grade.

NH₄Cl-washed ribosomes were prepared as described by Sander *et al.* (1975); 1 A_{260} unit was taken to represent 25 pmol of ribosomes. The tRNA_{1^{Val}} (Boehringer) was 95% pure; Val-tRNA_{1^{Val}} was prepared as described by Sander *et al.* (1975).

The GTPase activity was measured as previously reported (De Vendittis *et al.*, 1986). To check the effect of univalent or bivalent cations on the activity of GTPase^p, a sample of EF-G was extensively dialysed against 20 mM-Tris/HCl, pH 7.8, supplemented with 1 mM-dithiothreitol (TD buffer); chloride salts were used as sources of univalent and bivalent cations.

The structural changes induced by propan-2-ol on EF-G were investigated by comparing the spectroscopic data of EF-G determined in the absence or in the presence of the alcohol. In all experiments the concentration of the alcohol is expressed as % (v/v) and, unless otherwise indicated, EF-G was dissolved in TMAD buffer.

C.d. measurements were made at 20 °C in 0.5 cm quartz cells in a Jobin-Yvon Mark III spectropolarimeter. The c.d. data were expressed as ellipticity ([θ], degrees·cm²·dmol⁻¹), taking 110 as the average M_r of an amino acid residue.

The fluorescence measurements were performed at 25 °C, in a Hitachi-Perkin-Elmer spectrofluorimeter model 650-40 equipped with a recorder model 561. When ANS was used as a fluorescence probe, the excitation and the emission wavelengths were 360 and 510 nm respectively.

To determine the number of binding sites for ANS on EF-G, as well as the dissociation constant of the EF-G·ANS complex, the equation developed by Klotz *et al.* (1946) was applied. This equation has the form:

$$\frac{1}{N} = \left[\frac{K_D}{n} \times \frac{1}{(1-X)D} \right] + \frac{1}{n}$$

where N is the number of mol of bound ANS/mol of protein, n is the number of ANS-binding sites/protein molecule, K_D is the dissociation constant of the EF-G·ANS complex, D is the total ANS concentration, and X is the fraction of ANS bound. A plot of $1/N$ versus $1/(1-X)D$ yields values of both n and K_D . The data were treated by the method of least squares. The fraction of ANS bound was determined as described by Brand *et al.* (1967), from the expression:

$$X = \left(\frac{F_{\text{meas}}}{F_{\text{free}}} - 1 \right) / \left(\frac{F_{\text{bound}}}{F_{\text{free}}} - 1 \right)$$

where F_{free} is the fluorescence of free ANS measured in the absence of protein, F_{bound} is the fluorescence of totally bound ANS determined on addition of excess EF-G, and F_{meas} is the fluorescence of bound ANS measured at intermediate concentrations of EF-G. Other details are given in the legend to Fig. 3.

The fluorescence quenching of the tryptophan residues of EF-G caused by acrylamide or KI was measured by the procedures described by Lehrer (1971) and Eftink &

Ghiron (1976) respectively. The excitation and the emission wavelengths were 295 and 343 nm respectively. When acrylamide was used as a quencher, appropriate corrections were made for its absorbance at the excitation wavelength. EF-G was dissolved in the TD buffer; univalent and bivalent cations, as well as propan-2-ol, were added separately or in combination, in order to evaluate their individual contributions to the structural changes provoked on the factor. In the experiments where KI was used as a quencher, EF-G was dissolved in the TMAD buffer. The oxidation of I⁻ to I₂ was prevented by addition of Na₂S₂O₃; moreover, appropriate amounts of KCl were added to keep the ionic strength constant. Other details are indicated in the legends to Figs. 4 and 5.

Protein concentration was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

RESULTS

Ionic requirements for the expression of GTPase^p

The GTPase activities displayed by EF-Tu (Parmeggiani & Sander, 1981) and EF-1 (Crechet & Parmeggiani, 1986) are both dependent on the ionic conditions, and the same holds for GTPase^p of EF-G (Sander *et al.*, 1978). The regulation of EF-G GTPase^p by univalent and bivalent cations, alone or in combination, has been studied in the present work. When the individual effect of univalent cations was investigated, 1 mM-EDTA was added in the assay mixture in order to remove traces of Mg²⁺; under these conditions the univalent cations NH₄⁺, Li⁺, Na⁺, K⁺ and Cs⁺, in the concentration range 1–300 mM, did not stimulate the GTPase^p. Addition of 1 mM-Mg²⁺ (in the absence of EDTA) caused a stimulation of GTPase^p, and the order of effectiveness was NH₄⁺ $\approx$ Li⁺ > Cs⁺ $\approx$ K⁺ > Na⁺; the concentration of M⁺ required for reaching the optimum activity was in the range 40–75 mM (Fig. 1a).

Bivalent cations alone stimulated GTPase^p: 1 mM-Sr²⁺, 2–5 mM-Ca²⁺ and 1 mM-Ba²⁺ were by far more effective than Mg²⁺ and Mn²⁺ (Fig. 1b). In presence of 75 mM-NH₄⁺ the order and the extent of the stimulatory effect by bivalent cations were reversed, since Mg²⁺ and Mn²⁺ became much more effective than Ba²⁺, Ca²⁺ and Sr²⁺ (Fig. 1c).

Under the most favourable ionic conditions, that is 2 mM-Ca²⁺ or 40 mM-Li⁺ plus 1 mM-Mg²⁺, the pH optimum for GTPase^p was in the range 8.0–8.5.

GTPase^p activity assayed in presence of tRNA_{1^{Val}}, Val-tRNA_{1^{Val}} and fusidic acid

In order to evaluate the effect on GTPase^p of tRNA and aminoacyl-tRNA, which have been reported to be biological regulators of the ribosome-dependent EF-G GTPase (Parmeggiani & Sander, 1981), GTPase^p was assayed in the presence of 3 μ M-tRNA_{1^{Val}} or -Val-tRNA_{1^{Val}}. Kinetic measurements of GTPase^p, performed at 30 °C in TD buffer containing 75 mM-NH₄Cl supplemented with 1 mM- or 7 mM-MgCl₂, were identical with those performed in the absence of tRNA_{1^{Val}} or Val-tRNA_{1^{Val}}. Similarly, no effect was observed in presence of 1 mM-sodium fusidate, which is an inhibitor of GTPase^p (Bodley *et al.*, 1970).

Fig. 1. Effect of univalent (M^+) and bivalent (M^{2+}) cations on GTPase^p

The reaction mixture contained, in 50 μ l of TD buffer, 50 pmol of EF-G, 2.5 nmol of [γ - 32 P]GTP (sp. radioactivity 120 c.p.m./pmol) and 20% (v/v) propan-2-ol. After incubation for 45 min at 30 $^{\circ}$ C, the release of [32 P]P_i was evaluated as described in the Materials and methods section. Blanks run in the absence of EF-G were subtracted. (a) Effect of M^+ in the presence of 1 mM-Mg²⁺: NH₄⁺ (●), Li⁺ (○), Na⁺ (■), K⁺ (△) and Cs⁺ (□). (b) Effect of M^{2+} : Mg²⁺ (●), Ca²⁺ (○), Ba²⁺ (■), Mn²⁺ (□) and Sr²⁺ (△). (c) Effect of M^{2+} in the presence of 75 mM-NH₄⁺; same symbols as in (b). The percentage of substrate hydrolysed never exceeded 5% of the initial amount.

C.d. measurements

In order to investigate whether the triggering of the GTPase activity of EF-G by propan-2-ol was related to changes in the secondary or tertiary structure of the factor, c.d. measurements at 220 nm and 285 nm were performed on EF-G in the presence of increasing concentrations of the alcohol. The results, shown in Fig. 2, indicated that $[\theta]_{220}$ was not substantially affected by propan-2-ol up to a 30% concentration. However, $[\theta]_{285}$ increased considerably at increasing concentrations of the alcohol; the enhancement observed in the presence of 20% (v/v) propan-2-ol was not reproduced by 20% ethanol (results not shown) which was previously found to be a minor effector of GTPase^p (De Vendittis *et al.*, 1986). It is worth mentioning that the value of -9500 degrees $\cdot$ cm² $\cdot$ dmol⁻¹ determined for $[\theta]_{220}$ was similar to that previously reported (Brot *et al.*, 1972; Alakhov *et al.*, 1979).

Fig. 2. Dependence of the c.d. of EF-G on the concentration of propan-2-ol

The initial concentration of EF-G was 3.6 μ M in TMAD buffer. The indicated concentrations of propan-2-ol were obtained by the addition of appropriate volumes of 90% (v/v) propan-2-ol dissolved in TMAD buffer. Corrections were made for the dilution of EF-G. The ellipticity $[\theta]$ was measured at 220 nm (●) and at 285 nm (○) as described in the Materials and methods section. Blanks in the absence of EF-G were subtracted.

Effect of propan-2-ol on the fluorescence emission of EF-G $\cdot$ ANS

The fluorescence emission of EF-G $\cdot$ ANS was considerably decreased in the presence of 20% propan-2-ol, compared with that observed in absence of the alcohol; this is shown in Fig. 3(a) for a typical experiment in which the dye in increasing concentration was added to a fixed concentration of EF-G. Fig. 3(b) reports the Klotz plot (Klotz *et al.*, 1946; Brand *et al.*, 1967) derived from the analysis of the data obtained from fluorescence measurements of EF-G $\cdot$ ANS in the absence of propan-2-ol; Fig. 3(c) reports a similar plot derived from data obtained in presence of the alcohol. The number of binding sites for ANS on EF-G calculated from these plots (see the Materials and methods section) was 13 in the absence of propan-2-ol and 6 in the presence of 20% propan-2-ol; the dissociation constant of the complex EF-G $\cdot$ ANS was calculated as 8 μ M in the absence and 5 μ M in the presence of the alcohol.

Quenching of the intrinsic fluorescence of EF-G by acrylamide or KI

The EF-G molecule contains six tryptophan residues (Ovchinnikov *et al.*, 1982; Kazirol *et al.*, 1972; Rohrbach *et al.*, 1975). We have investigated the exposure of these aromatic residues by measuring the quenching of the intrinsic fluorescence of the factor caused by a neutral or charged quencher.

Fig. 3. Fluorescence of EF-G·ANS in the presence and in the absence of propan-2-ol

The experiments were performed at room temperature with excitation and emission wavelengths of 360 and 510 nm respectively. Increasing concentrations of ANS were obtained by adding suitable amounts of a 100 μM stock solution in TMAD buffer. (a) Effect of propan-2-ol on the fluorescence intensity of EF-G·ANS. ANS at increasing concentrations, up to 12 μM, was added to 1.25 μM-EF-G dissolved in TMAD buffer in the absence (●) or the presence (○) of 20 % propan-2-ol. (b) Klotz plot for determining the number of ANS-binding sites (n) on EF-G and the dissociation constant of the EF-G·ANS complex in the absence of propan-2-ol. ANS at increasing concentration, up to 9 μM, was added to 0.25 μM- (▲) and 0.63 μM- (●) EF-G solutions. F_{bound} was measured in presence of an excess of EF-G, equal to 1.25 μM (see the Materials and methods section). (c) Klotz plot for determining the number of ANS-binding sites (n) on EF-G and the dissociation constant of the EF-G·ANS complex in presence of 20 % propan-2-ol. ANS at increasing concentration, up to 12 μM, was added to 1.26 μM- (○) and 1.89 μM- (△) EF-G. F_{bound} was measured in presence of an excess of EF-G equal to 2.5 μM (see the Materials and methods section). In all cases fluorescence measurements were corrected for the dilution of EF-G and for fluorescence of ANS alone at each selected concentration of the dye.

Fig. 4. Quenching of the intrinsic fluorescence of EF-G by acrylamide in the absence or the presence of propan-2-ol

EF-G (2.1 nmol) was dissolved in 600 μl of TD buffer in the absence (●) or in the presence (○) of 20 % propan-2-ol. The A_{295} was in both cases 0.1. The indicated concentrations of acrylamide were obtained by sequential additions of small amounts from a 8 M stock solution prepared in TD buffer in the absence or presence of 20 % propan-2-ol. Corrections were made for the dilution of EF-G and for the absorption owing to acrylamide. The fluorescence measurements were performed as indicated in the Materials and methods section. Blanks in the absence of EF-G were subtracted.

Fig. 5. Quenching of the intrinsic fluorescence of EF-G by KI in the absence or the presence of propan-2-ol

Two samples of EF-G (3.6 nmol) were prepared in 1 ml final volume of TMAD buffer supplemented with 0.5 mM- $\text{Na}_2\text{S}_2\text{O}_3$ and containing 400 mM-KI or 400 mM-KCl, respectively. The indicated concentrations of KI were obtained by mixing appropriate volumes of the EF-G solutions prepared in the presence of KI or KCl to keep a constant ionic strength. Fluorescence quenching was measured on EF-G solutions prepared in the absence (●) or the presence (○) of 20 % propan-2-ol.

In Fig. 4 the Stern-Volmer plot obtained by using acrylamide as a neutral quencher is reported. The addition of 20 % propan-2-ol in TD buffer increased the quenching of fluorescence of the tryptophan residues of

EF-G; furthermore, the linearity of the plot suggests that under this condition tryptophan residues are equally exposed to the solvent. This behaviour was also observed with propan-2-ol in presence of 2 mM- Ca^{2+} or 1 mM- Mg^{2+} plus 75 mM- NH_4^+ , thus showing that a different

Fig. 6. Time courses of GTPase' and GTPase^P at different temperatures

(a) Rate of GTP hydrolysis in the GTPase' system (●) at the indicated temperatures. The reaction mixture contained, in 270 μl of TD buffer, 10 mM-Mg²⁺, 80 mM-NH₄⁺, 180 pmol of ribosomes, 45 pmol of EF-G and 13.5 nmol of [γ -³²P]GTP (sp. radioactivity 48 c.p.m./pmol). After incubation, 60 μl samples were withdrawn at the indicated times and analysed for [³²P]P_i released as described in the Materials and methods section. (b) Rate of GTP hydrolysis in the GTPase^P system (○) at the same temperatures as for GTPase'. The reaction mixture contained, in 270 μl of TD buffer, 1 mM-Sr²⁺, 225 pmol of EF-G, 11.3 nmol of [γ -³²P]GTP (sp. radioactivity 53 c.p.m./pmol) and 20 % propan-2-ol. After incubation, 60 μl samples were withdrawn at the indicated times and analysed for [³²P]P_i released as described in the Materials and methods section. Blanks run in the absence of EF-G were subtracted. (c) Temperature-dependence of the rates of GTPase' and GTPase^P. The natural logarithm of the slope of the kinetics shown in (a) and (b) is plotted versus the reciprocal of the absolute temperature.

ionic environment does not change the effect of the alcohol.

In order to investigate whether or not the effect of propan-2-ol depended on the quencher used, acrylamide was replaced by KI as a charged quencher. The results, shown in Fig. 5, were essentially similar to those obtained with the neutral acrylamide. When propan-2-ol was replaced by glycerol the Stern-Volmer plots were in both cases almost coincident with those obtained in the absence of the alcohol, thus indicating that changes in the conformation of EF-G are specifically provoked by propan-2-ol. This finding is in agreement with the fact that 20 % glycerol is almost ineffective in stimulating GTPase activity of EF-G in the absence of ribosomes (De Vendittis *et al.*, 1986).

Temperature-dependence of GTPase^P

The effect of temperature on the GTPase^P reaction was studied under the best ionic condition previously determined, i.e. 1 mM-Sr²⁺ (see Fig. 1); a comparison was then made with GTPase' assayed in the presence of 80 mM-NH₄⁺ plus 10 mM-Mg²⁺ (Sander *et al.*, 1978). The time course of activity measured at 20, 25, 30 and 35 °C is shown in Fig. 6(a) for GTPase' and in Fig. 6(b) for GTPase^P. At each selected temperature the kinetics started from the origin and were thoroughly linear. Under the conditions employed, at each temperature the amount of GTP hydrolysed by EF-G in 1 min was about three orders of magnitude higher for GTPase' than for GTPase^P, thus confirming a previous finding (De Vendittis *et al.*, 1986).

In Fig. 6(c) the natural logarithm of the slope of the kinetics related to either GTPase' or GTPase^P is plotted versus the reciprocal of the absolute temperature. The two straight lines obtained had an almost identical slope, thus suggesting that GTPase' and GTPase^P had also

similar energetic requirements when assayed under the most convenient ionic conditions. Similar results were obtained when GTPase^P was measured in the presence of 2 mM-Ca²⁺ or 1 mM-Mg²⁺ supplemented with 75 mM-NH₄⁺ or 40 mM-Li⁺.

DISCUSSION

In the course of the translocation process EF-G displays a GTPase activity which requires the presence of several effectors, such as univalent and bivalent cations, aminoacyl-tRNA, mRNA and ribosomes (Miller & Weissbach, 1977; Kaziro, 1978; Parmeggiani & Sander, 1981). In an earlier paper (De Vendittis *et al.*, 1986) we reported that, in a simplified system uncoupled from protein synthesis, EF-G can display a GTPase activity triggered by propan-2-ol (GTPase^P) in the absence of ribosomes. This finding has allowed us to attribute to EF-G the site for the catalytic hydrolysis of GTP, a property which was already well established for the elongation factor Tu (Parmeggiani & Sander, 1981).

In the present paper the ionic requirements for the optimal expression of GTPase^P were investigated (Fig. 1). The simplest ionic environment was obtained in presence of the bivalent cations Sr²⁺ (1 mM), Ca²⁺ (2–5 mM) or Ba²⁺ (1 mM). Another condition was a combination of 1 mM-Mg²⁺ plus 40–70 mM-Li⁺ or -NH₄⁺. These ionic conditions were compared with those reported for another GTPase system also uncoupled from protein synthesis, that is EF-Tu·kirromycin GTPase, in which the antibiotic makes EF-Tu capable of hydrolysing GTP even in the absence of the physiological effectors aminoacyl-tRNA and ribosomes (Wolf *et al.*, 1974). The first observation resulting from such a comparison is that in absence of free Mg²⁺ (1 mM-EDTA) univalent cations up to 300 mM do not stimulate GTPase^P, whereas in the

presence of 200 mM-NH₄⁺ a measurable GTPase activity of EF-Tu·kirromycin was detected (Ivell *et al.*, 1981). This might indicate that univalent cations do not interact with the catalytic centre of EF-G that is activated by propan-2-ol. In presence of 1 mM-Mg²⁺ the effect of M⁺ on GTPase^p reaches a maximum at about 40–75 mM-M⁺ (Fig. 1*a*), whereas in the EF-Tu·kirromycin system the GTPase activity increases continuously up to 2 M-M⁺ (Ivell *et al.*, 1981). In the GTPase^p system, bivalent cations do not require the presence of univalent cations and the order of effectiveness is Sr²⁺ > Ca²⁺ > Ba²⁺ > Mn²⁺ > Mg²⁺ (Fig. 1*b*); the addition of 75 mM-NH₄⁺ increases considerably the effect of Mn²⁺ and Mg²⁺ and decreases dramatically the effect of Sr²⁺, Ca²⁺ and Ba²⁺, so that the order of effectiveness becomes the following: Mn²⁺ ≈ Mg²⁺ > Ba²⁺ ≈ Ca²⁺ ≈ Sr²⁺ (Fig. 1*c*). In presence of 200 mM-NH₄⁺ the GTPase activity of EF-Tu·kirromycin was strongly stimulated by Mn²⁺, followed far behind by Ba²⁺, Sr²⁺, Ca²⁺ and Mg²⁺ (Ivell *et al.*, 1981). These results indicate that univalent cations greatly modulate the influences of M²⁺ for the stimulation of the active site of EF-G induced by propan-2-ol, whereas in the EF-Tu·kirromycin system the addition of 200 mM-NH₄⁺ makes only Mn²⁺ much more effective than other M²⁺ species (Ivell *et al.*, 1981). From all these observations it emerges that the ionic requirements for the expression of GTPase^p of EF-G differ from those reported for the GTPase activity of EF-Tu·kirromycin.

In the course of the present work we found that the addition of tRNA_{1^{val}} or Val-tRNA_{1^{val}} had no effect on the activity of GTPase^p, thus suggesting that no direct interaction occurs between EF-G and acylated or deacylated tRNA; in contrast, acylated and deacylated tRNA are effectors of GTPase^r (Parmeggiani & Sander, 1981). Similarly fusidic acid, an inhibitor of the turnover of GTPase^r (Bodley *et al.*, 1970), did not affect GTPase^p.

To see whether the triggering by propan-2-ol of GTPase^p was accompanied by structural changes of EF-G, c.d. and fluorescence measurements were carried out. The experiment reported in Fig. 2 showed that propan-2-ol considerably increased the ellipticity of EF-G measured at 285 nm; in contrast, the value of $[\theta]_{220}$ remained unchanged. This suggests that propan-2-ol alters the tertiary structure of EF-G without affecting its secondary structure. The increase in $[\theta]_{285}$ provoked by propan-2-ol on EF-G was related to the appearance of a distinct peak at 287 nm, which was not detected in the absence of the alcohol (Parlato *et al.*, 1985). The effect of propan-2-ol on the ellipticity of EF-G increased with the concentration of the alcohol. This finding was evaluated together with the activation of the catalytic site of EF-G with increasing concentration of propan-2-ol (De Vendittis *et al.*, 1986). Moderate alterations in the structure of EF-G induced by the alcohol up to 20% concentration provoke the activation of the catalytic site for GTP hydrolysis. It has to be emphasized that the effect of propan-2-ol on the c.d. spectra of EF-G is specific, since 20% ethanol, a minor effector of GTPase^p (De Vendittis *et al.*, 1986), did not significantly alter the value of $[\theta]_{285}$.

The fluorescence experiments performed with ANS (Fig. 3) showed that propan-2-ol decreased the fluorescence of the dye bound to EF-G. The decrease in the dissociation constant of the EF-G·ANS complex found in the presence of propan-2-ol, accompanied by a diminution of the number of ANS molecules bound per

molecule of EF-G, suggests a disruption by propan-2-ol of hydrophobic domains in the protein (Singer, 1962).

The results of fluorescence-quenching experiments with non-ionic (acrylamide) and ionic (I⁻) quenchers suggest that propan-2-ol induces conformational changes on EF-G, resulting in the exposure of tryptophan residues. When acrylamide was used as a quencher (Fig. 4), it was found that the Stern–Volmer plot in the absence of propan-2-ol curved downward. On addition of 20% propan-2-ol the Stern–Volmer plot became linear, thus indicating an equal exposure of tryptophan residues in the EF-G molecules (Eftink & Ghiron, 1976). An alteration of the EF-G structure by the quencher itself can be excluded, since the increased exposure of the tryptophan residues in the presence of propan-2-ol was observed also with KI (Fig. 5). Therefore it is likely that propan-2-ol affects the tertiary structure of EF-G, at least in those regions containing tryptophan residues. The enhancement of GTPase activity of EF-G by propan-2-ol may then be related to a moderate denaturation of EF-G. Activation of enzymes after treatment with mild denaturing agents has been reported previously (Bocchini *et al.*, 1967; Bocchini & Najjar, 1970).

To investigate whether the conformational changes in EF-G induced by propan-2-ol, revealed by fluorescence and c.d. studies, were followed by further changes, time-dependent or temperature-dependent, the kinetics of GTPase^p was followed in the range 20–35 °C (up to 60 min at 20 °C) and a comparison was made with GTPase^r. Figs. 6*(a)* and 6*(b)* show that at each selected temperature the kinetics was linear for both systems, and suggest that conformational modifications of EF-G induced by propan-2-ol were not followed in the time by further changes capable of modifying the GTPase activity. Similarly, the plot shown in Fig. 6*(c)* derived from a kinetic analysis of the data reported in Fig. 6*(b)* suggests that conformational modifications provoked on EF-G by propan-2-ol were not followed by temperature-dependent changes in the structure of EF-G. Finally, the fact that the two plots shown in Fig. 6*(c)* are almost parallel indicates that the energetic requirements of GTPase^p do not differ significantly from those related to GTPase^r, although the activity of the latter is about three orders of magnitude higher than that for GTPase^p (De Vendittis *et al.*, 1986). These results indicate that, in the absence of ribosomes, propan-2-ol induces on EF-G a conformation which makes the active site accessible to the substrate. More extensive studies are needed to evaluate the effect of M⁺ and M²⁺ on the energetic and entropic parameters of GTPase^p.

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