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Modulation by Monovalent and Divalent Cations of the Guanosine-5′-triphosphatase Activity Dependent on Elongation Factor Tu†

Richard Ivell,‡ Gernot Sander,§ and Andrea Parmeggiani*

ABSTRACT: In this work, the role of monovalent (M^+) and divalent (M^{2+}) cations in the GTPase activity of elongation factor Tu (EF-Tu) is studied in systems of increasing complexity. The GTPase activity induced by kirromycin in the absence of aminoacyl-tRNA (aa-tRNA) and ribosomes requires M^+; it increases with increasing [M^+] and above 0.4 M M^+ becomes inversely proportional to the cationic radius, the order of effectivity thus being $Li^+ > Na^+ > K^+ > NH_4^+ > Cs^+ > Me_4N^+$. Its K_m is similar for all M^+ species at 0.2 M M^+ (0.2 μM) but increases in the order of cation effectivity at 2 M M^+ (up to 1.1 μM for Li^+). Addition of aa-tRNA and/or ribosomes to this system stimulates the GTPase activity at [M^+] < 0.8 M but has little effect at higher [M^+]. With both aa-tRNA and ribosomes, maximum stimulation occurs in the absence of added M^+, except for NH_4^+, whose optimum concentration is 250 mM. Thus, in the kirromycin system, increasing [M^+] can substitute for the effect of aa-tRNA and ribosomes, suggesting that the action of these components is of ionic type. In the absence of the antibiotic, in a more specific system containing Phe-tRNA^{Phe}, ribosome·poly(U), and elongation factor Ts (physiological system), the M^+ are generally inhibitory, the K_m of the reaction corresponding to the highest K_m values observed in the kirromycin system for the respective cations. The EF-Tu·kirromycin GTPase does not need free Mg^{2+} or nucleotide-bound Mg^{2+}; nevertheless, the large excess of EDTA required to eliminate the activity suggests that a tightly bound M^{2+} is essential for the active conformation of EF-Tu·kirromycin. However, neither Mg^{2+} nor Ca^{2+} or Mn^{2+} was found to be tightly bound to EF-Tu. Without free Mg^{2+}, there is a marked interdependence between [M^+] and pH in the optimum of the EF-Tu·kirromycin GTPase activity, indicative of an anionic region close to and intimately involved in the catalytic center for GTP hydrolysis on the factor. This anionic region appears to be masked by added Mg^{2+}. At 0.2 M M^+, ribosomes show a specific stimulation dependent on free Mg^{2+} of the EF-Tu·kirromycin GTPase activity, whereas at 2 M M^+, they appear to display an effect dependent solely on bound Mg^{2+}. In the kirromycin system, Mg^{2+} can be effectively replaced by the class 2A metals (Be^{2+} excepted) at low and high [M^+]. In the physiological GTPase activity, M^{2+} are absolutely required and are effective in the order $Mn^{2+} > Ca^{2+} = Ba^{2+} = Sr^{2+} > Mg^{2+}$. When their concentrations are increased, particularly that of Mg^{2+}, the requirement of the EF-Tu GTPase activity for codon–anticodon interaction is partially or wholly eliminated, suggesting an important role for divalent cations in the recognition process.

During the tRNA[1]-mediated translation of mRNA into polypeptide, elongation factor Tu (EF-Tu) is responsible for the specific emplacement of an aminoacylated tRNA into the A site of the ribosome. After the binding of the ternary complex EF-Tu·GTP·aa-tRNA to the ribosome and the associated hydrolysis of GTP, EF-Tu·GDP leaves the ribosome, thereby allowing the peptide bond formation [for reviews, see Lucas-Lenard & Beres (1974), Miller & Weissbach (1977) and Bermek (1978)]. To be recycled, the complex EF-Tu·GDP interacts with a second elongation factor, EF-Ts, which accelerates the rate of formation of the complex EF-Tu·GTP. This is then stabilized by the binding of a new molecule of aa-tRNA (Fasano et al., 1978).

The antibiotic kirromycin, which binds to EF-Tu in a stoichiometric ratio of 1:1 [Chinali et al., 1977; for a review, see Parmeggiani & Sander (1980)], modifies the conformation of EF-Tu·GDP, such that, even though the fast hydrolysis of GTP occurs nomally, EF-Tu·GDP remains fixed onto the

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[1] Abbreviations used: EF-Tu, elongation factor Tu; EF-Ts, elongation factor Ts; tRNA, transfer ribonucleic acid; tRNA^{Phe}, phenylalanine-accepting tRNA; Phe-tRNA^{Phe}, tRNA chared to 40–50% with phenylalanine; aa-tRNA, aminoacyl-tRNA; mRNA, messenger RNA; M^+, monovalent cations; M^{2+}, divalent cations; GTP, guanosine 5′-triphosphate; GDP, guanosine 5′-diphosphate; GTPase, guanosine-5′-triphosphatase; GTPase/enzyme, EC 3.6.1; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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ribosome·mRNA, effectively preventing peptide bond formation (Wolf et al., 1977). EF-Tu·kirromycin is capable of hydrolyzing GTP with the properties of a turnover reaction in the absence of aa-tRNA and ribosomes; these two components are able to stimulate the activity induced by the antibiotic (Wolf et al., 1974). Recently, we have found that the GTPase activity of EF-Tu·kirromycin depends on the presence of monovalent cations (Sander et al., 1975).

In order to understand better the functional organization of the EF-Tu molecule, we have studied the influence of monovalent and divalent cations of the GTPase activity of EF-Tu in both the kirromycin-dependent and physiological systems. In particular, we have investigated their importance in the interactions between the factor, ribosomes, and aa-tRNA, their involvement in the active site for GTP hydrolysis, and the effect of cation species per se.

Materials and Methods

All biological components, materials, and methods not mentioned in this section were as already reported (Sander et al., 1975; Chinali et al., 1977; Fischer et al., 1977). Kirromycin (=mocimycin) was a gift of Drs. R. Beukers (Gyst-Brocades, Delft) and H. Wolf (Institute of Microbiology, University of Tübingen).

The NH_4Cl-washed ribosomes (Sander et al., 1975) were dialyzed with several changes against a buffer containing 50% glycerol, 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, and 60 mM of an appropriate M^+ and stored at −25 °C. Thus addition of ribosomes to an assay did not introduce foreign M^+ where a specific M^+ was to be tested, except possibly where the ions were tightly bound. These ribosomes comprised at least 80% tight couples as assessed by sucrose density centrifugation in 5 mM $MgCl_2$ and retained maximal activity in poly(U)-directed poly(phenylalanine) synthesis for several weeks, with $[Mg^{2+}]$ optima below 10 mM.

Ribosomes were incubated exactly as in normal assays for 10 min at 30 °C in the presence of 2 M Li^+ and then precipitated with 2 volumes of ethanol to check the effect on ribosomes of high $[M^+]$. After centrifugation for 20 min at 10000*g*, the pelleted ribosomes were redissolved in a buffer containing 10 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.8, and 60 mM NH_4Cl and recentrifuged at 100000*g* for 4 h. Aliquots of the final pelleted ribosomes were then analyzed by two-dimensional electrophoresis (Kaltschmidt & Wittmann, 1970) and missing proteins identified.

Electrophoretically pure, crystalline EF-Tu from *Escherichia coli* BT2^r, prepared as described (Chinali et al., 1977), was dialyzed with several changes against a buffer containing 50% glycerol, 5 mM $MgCl_2$, 25 mM imidazolium–acetate, pH 7.5, 25 mg/L phenylmethanesulfonyl fluoride, and 10 mM GDP and stored at −25 °C. In this form, the factor was free of M^+ and remained stable and active for at least several months. The EF-Tu concentration was checked both by the method of Lowry et al. (1951) and by measuring maximum binding of EF-Tu·[^{3}H]GDP on nitrocellulose filters (Miller & Weissbach, 1977), which gave similar values.

Assay for GTPase Activity. The hydrolysis of [γ-^{32}P]GTP was measured as liberation of inorganic phosphate, and extraction was with isopropyl acetate in the presence of sodium molybdate (Sander et al., 1975). When cations interfered with this reaction, after the assay was stopped with $HClO_4$ as above, all nucleotides were sequestered by the addition of 0.4 mL of 6% activated charcoal in 1 M HCl (Donner et al., 1978), with only the $^{32}P_i$ liberated by hydrolysis remaining in solution. After centrifugation for 40 min at 5000 rpm, 0.2-mL aliquots of the supernatant were added to 5 mL of Aquasol (New England Nuclear) and counted in an Intertechnique SL4000 liquid scintillation counter. All assays were performed in a buffer containing 50 mM imidazolium acetate, pH 7.5, which decreased by less than 0.3 pH unit between 0 and 2 M M^+. Where ribosomes were included in the assays, minimum $[M^+]$ achieved were 0.3–1 mM because of carry-over with the ribosomes; otherwise, the minimum values were less than 0.03 mM. In the presence of kirromycin, reaction mixtures were incubated for 10 min at 30 °C, during which prior kinetic studies showed the reaction to proceed linearly with time [see also Bocchini et al. (1980)]. Incubation in the absence of the antibiotic was for 5 min at 30 °C; although this reaction is nonlinear, kinetic studies indicated less than 15% departure from linearity up to this time in the chosen assay conditions. All other data concerning reaction mixtures are indicated in the figure legends.

Results

Effect of Monovalent Cations on the EF-Tu·Kirromycin GTPase. With EF-Tu only in the presence of kirromycin, the simplest system giving a turnover GTPase activity in the absence of aa-tRNA and ribosomes, monovalent cations are an absolute requirement (Figure 1, dashed line). In general, an increase in $[M^+]$ brings about higher GTPase activity. At concentrations higher than 0.4 M M^+, the extent of this stimulation depends upon cation species and follows the order $Li^+ > Na^+ > K^+ > NH_4^+ > Cs$; Me_4N^+ and Tris·H^+ do not stimulate, even at 2 M concentration. At lower $[M^+]$, the differences between the active cations are less pronounced, K^+ and NH_4^+ being, however, the two most efficient followed by Na^+ and Li^+. In the presence of NH_4^+, EF-Tu·kirromycin GTPase activity levels off at between 0.4 and 1 M, whereas for Li^+, stimulation continues up to a maximum at about 3.5 M. At 5 M there is almost complete inhibition (not shown).

Below about 0.5 M M^+, both ribosomes and aa-tRNA, individually or together (Figure 1, solid lines), stimulate the hydrolysis of GTP in a manner characteristic of the monovalent cation species. Maximum stimulation by aa-tRNA plus ribosomes occurs without added M^+, except in the case of NH_4^+, whose optimum concentration is 0.25 M. Increasing $[M^+]$ reduces the stimulatory effect. At 2 M M^+, addition of ribosomes and/or aa-tRNA little modifies the pattern observed in their absence. The decreasing stimulatory effect of ribosomes at these high $[M^+]$ may be due in part to an inactivation of ribosomal functions resulting from the loss of component proteins. In fact, with ribosomes incubated under the same conditions in 2 M Li^+, we observed on bidimensional polyacrylamide gel electrophoresis (Kaltschmidt & Wittmann, 1970) that ribosomal proteins S2, S3, S5, S13, S14, S21, L1, L7/L12, L10, L16, and L25 were partially or wholly removed from the ribosome, though still present in solution.

Interestingly, at 0.2 M M^+, the K_m values of the GTPase activity of EF-Tu·kirromycin are all about 0.2 μM, with little, if any, cation specific variations, whereas at 2 M M^+, there is a large cation-dependent variation in the K_m from 0.2 μM for NH_4^+, the only cation inducing the same K_m values as at the 200 mM concentration, to 1.0 μM for Li^+ (Table I). The presence of ribosomes and aa-tRNA does not essentially change the K_m values at either 200 mM or 2 M M^+; only with NH_4^+ is the K_m twice as high [Table I; see also Bocchini et al. (1980)].

Influence of Monovalent Cations on the EF-Tu GTPase in the Absence of Kirromycin. In addition to the GTP hydrolysis accompanying the enzymatic binding of aa-tRNA to the ribosome·mRNA, a turnover GTPase activity can also be observed in the system lacking kirromycin, particularly in the

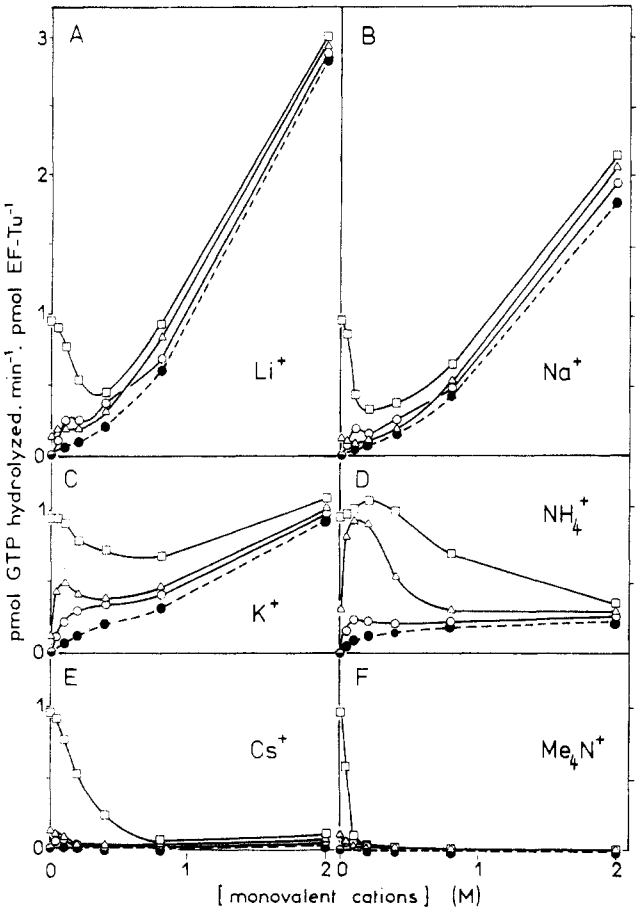

FIGURE 1: Effect of monovalent cations on the EF-Tu GTPase in the presence of kirromycin plus and minus ribosomes and/or aa-tRNA. Reaction mixtures contained in a volume of 75 μL, 10 mM $MgCl_2$, 50 mM imidazolium acetate, pH 7.5, 20 pmol of EF-Tu, 2 nmol of [γ-^{32}P]GTP (specific activity 100–150 cpm/pmol), 50 μM kirromycin, and monovalent cations as indicated: (A) Li^+, (B) Na^+, (C) K^+, (D) NH_4^+, (E) Cs^+, and (F) Me_4N^+. Without (●) or with (○) the addition of 100 pmol of Phe-tRNA^{Phe}, 40 pmol of ribosomes (△), or both Phe-tRNA^{Phe} and ribosomes (□).

Table I: K_m Values in the EF-Tu·Kirromycin GTPase Activity in the Absence of aa-tRNA and Ribosomes (Minus) and in Their Presence (Plus) as a Function of [M^+]

	K_m (μM)					
	10 mM		200 mM		2 M	
cations	minus	plus	minus	plus	minus	plus
Li^+		0.25	0.20	0.20	1.10	1.10
Na^+		0.25	0.15	0.15	0.80	1.00
K^+		0.40	0.20	0.30	0.50	0.60
NH_4^+		0.40	0.20	0.40	0.20	0.30
Cs^+		0.35	0.20	0.20	0.40	0.40
Me_4N^+		0.25	0.20	0.20		

presence of EF-Ts which by loosening the tightly associated EF-Tu·GDP encourages the recycling of EF-Tu. So that the effect of M^+ on this system could be tested, curves of GTPase activity vs. increasing [M^+] were constructed, starting the reaction with EF-Tu·GDP (Figure 2). Except for EF-Tu, all other components were in excess. In general, above 30 mM M^+, there is a steady decline in activity with increasing concentration for all cation species, reaching low activity levels at 0.4 M M^+. There is a significant cation specificity: at 30 mM M^+, Na^+ and Li^+ encourage a GTPase activity more than double that with NH_4^+; at 2 M M^+, with Li^+, K^+, and Na^+, one still observes a significant activity over the background levels.

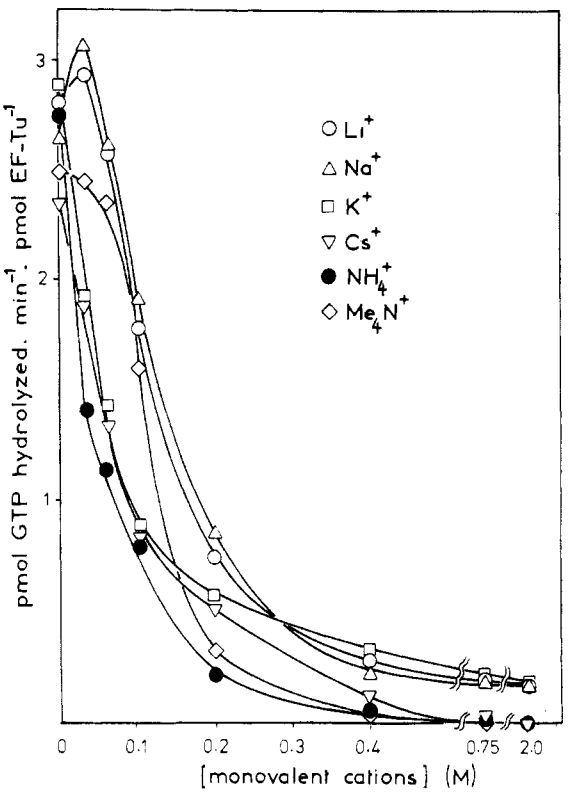

FIGURE 2: Effect of monovalent cations on the EF-Tu GTPase induced by aa-tRNA, mRNA, ribosomes, and EF-Ts. Reaction mixtures contained, in a volume of 75 μL, 10 mM $MgCl_2$, 50 mM imidazolium acetate, pH 7.5, 10 pmol of EF-Tu, 40 pmol of EF-Ts, 1 or 2 nmol of [γ-^{32}P]GTP (specific activity 100–200 cpm/pmol), 4 μg of poly(U), 100 pmol of Phe-tRNA^{Phe}, 20 pmol of ribosomes, and monovalent cations as indicated: (○) Li^+, (△) Na, (□) K^+, (●) NH_4^+, (▽) Cs^+, and (◇) Me_4N^+.

The K_m values were determined with all cations at 30 mM concentration. Except for NH_4^+, the kinetics at the different GTP concentrations were nonlinear, and initial velocities were estimated from the GTP hydrolyzed in the first 30 s of the reaction. In the presence of NH_4^+, the K_m was approximately 0.3 μM, a value similar to that calculated at low [M^+] in the kirromycin system (Table I and text of the relative section). The other cations all yielded K_m values between 0.5 and 1 μM, similar to the maximum values attained in the kirromycin system at 2 M M^+. The highest value was recorded for Na^+ (K_m = 1.0 μM).

Effect of Monovalent Cations on the Mg^{2+} Requirement of the EF-Tu·Kirromycin GTPase Activity. At 0.2 M M^+, Mg^{2+} appears to have only slight influence on the GTPase activity of EF-Tu·kirromycin alone; values in the absence of added Mg^{2+} and in the presence of 0.5 mM EDTA are only somewhat lower than those at 10 or 20 mM Mg^{2+} (Figure 3A). This pattern is parallel for all M^+ and suggests that in this condition free Mg^{2+} can be adequately replaced by a variety of M^+.

At high [M^+] (2 M), however (Figure 3C), M^{2+} stimulates activity up to 15-fold, though it is important to note that also in this case in the absence of Mg^{2+} (0.5 mM EDTA) activity is still present. Under these high ionic strength conditions, M^+ can evidently not completely replace Mg^{2+} ions.

At 0.2 M M^+, there is a strong M^+-specific stimulation by ribosomes of the EF-Tu·kirromycin GTPase activity (Figure 3B). This effect is clearly Mg^{2+} dependent, being greatest for NH_4^+, followed by K^+ and lastly by Li^+; in the presence of Na^+, Cs^+, and Me_4N^+, there is scarcely any ribosomal stimulation. The effect is additive to the Mg^{2+}-independent activity

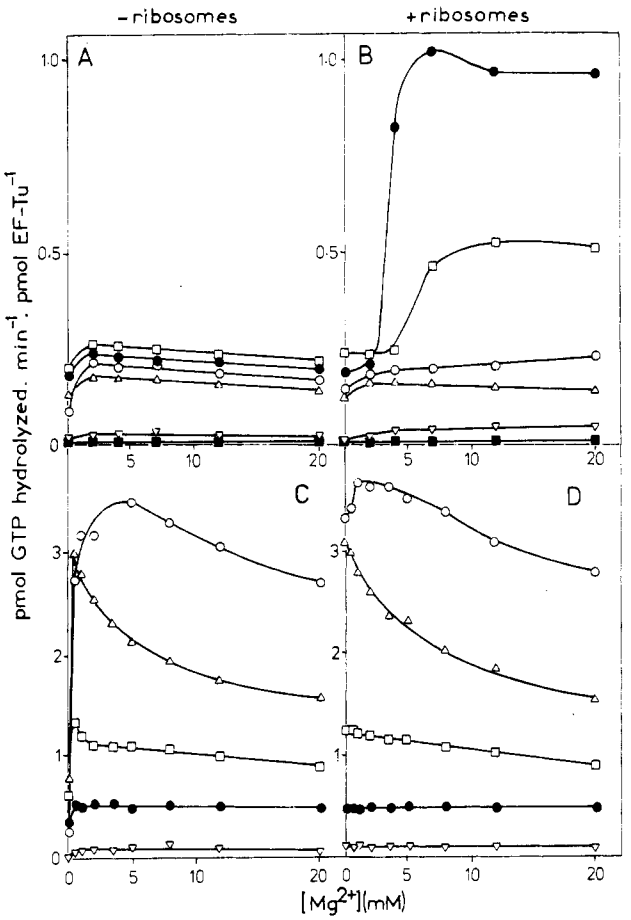

FIGURE 3: Effect of Mg^{2+} on the EF-Tu·kirromycin GTPase in the presence of low and high concentrations of monovalent cations plus or minus ribosomes. Reaction mixtures contained, in 75 μL, 50 mM imidazolium acetate, pH 7.5, 50 μM kirromycin, 2 nmol of [γ-^{32}P]GTP (specific activity of 100–150 cpm/pmol), 20 pmol of EF-Tu, 40 pmol of ribosomes in (B) and (D), and $MgCl_2$ as indicated. (○) Li^+, (△) Na^+, (□) K^+, (●) NH_4^+, (▽) Cs^+, and (■) Me_4N^+. A,B: 200 mM M^+; C,D: 2 MM^+.

present in the absence of ribosomes and only occurs at $[Mg^{2+}]$ higher than about 4 mM.

At 2 M M^+, ribosomes only affect the picture observed in their absence (Figure 3B) by eliminating entirely the requirement for Mg^{2+} to induce maximum activity (Figure 3D). This suggests that the ribosomes are still influencing the activity via bound Mg^{2+}. In experiments not shown, this effect was a property only of the 50S ribosomal subunits, 30S subunits being without influence, implying an unexpected interaction specificity at such high $[M^+]$.

Specificity of Divalent Cations in the EF-Tu·Kirromycin GTPase. At 0.2 M NH_4^+ and 5 mM M^{2+}, in the presence of EDTA (0.5 mM), only the class IIA metals (alkaline earths) (Be^{2+} excepted) and Mn^{2+} stimulate the basal GTPase activity of EF-Tu·kirromycin in the order $Mn^{2+} > Ba^{2+} > Sr^{2+} > Ca^{2+} > Mg^{2+}$. All other divalent metal ions were strongly inhibitory (Figure 4). At 2 M Li^+, similar results were obtained, the class IIA metals (Be^{2+} excepted) and Mn^{2+} stimulating; Co^{2+}, however, and to a lesser extent Fe^{2+}, which at 0.2 M NH_4^+ inhibited, here also showed stimulatory activity. The order of effectivity was $Mg^{2+} > Mn^{2+} > Ca^{2+} > Sr^{2+} > Ba^{2+} > Co^{2+}$. With the exception of Fe^{2+}, no enzymatic hydrolysis of GTP, catalyzed directly by the metal ions (Amsler & Sigel, 1976), was observed.

Concentration curves were made for the class 2A metal ions (Be^{2+} excepted) and Mn^{2+} at 0.2 M NH_4^+ and 2 M Li^+ (Figure 5). In the former conditions, the larger the class IIA

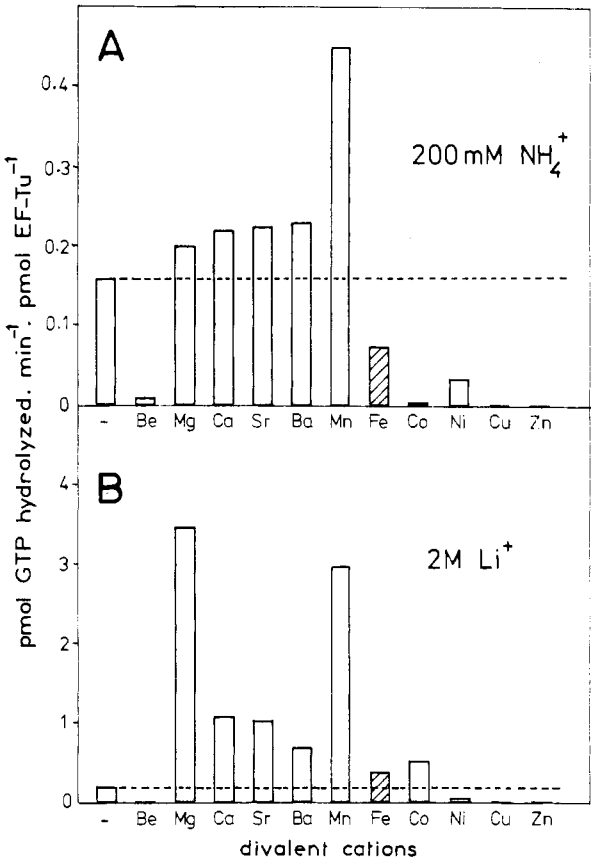

FIGURE 4: Effect of the various divalent cations on the EF-Tu·kirromycin GTPase. Reaction mixtures contained, in 75 μL, 5 mM M^{2+} Cl, M^+ as indicated, 50 mM imidazolium acetate, pH 7.5, 0.5 mM Tris·H^+EDTA, pH 7.5, 20 pmol of EF-Tu, 50 μM kirromycin, and 1 or 2 nmol of [γ-^{32}P]GTP (specific activity 100–200 cpm/pmol). (A) At 200 mM NH_4Cl; (B) at 2 M LiCl. The hatched bar for Fe^{2+} indicates that this value did not differ from the already high blank in the absence of EF-Tu.

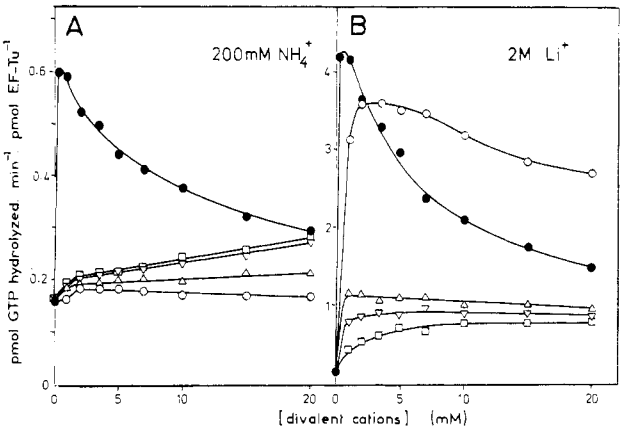

FIGURE 5: Concentration curves for the effect of divalent cations on the EF-Tu·kirromycin GTPase. Reaction mixtures contained 0.5 mM Tris·H^+EDTA, pH 7.5, and M^{2+} as indicated; other conditions as in Figure 4. (○) Mg^{2+}, (△) Ca^{2+}, (▽) Sr^{2+}, (□) Ba^{2+}, and (●) Mn^{2+}. (A) At 200 mM NH_4Cl; (B) at 2 M LiCl.

ion, the greater the stimulatory activity. The Mg^{2+} was therefore the least active and Ba^{2+} the most active. The activity of Mn^{2+} was even greater, and in its presence, the GTPase activity displayed a sharp optimum below 0.2 mM. At 2 M Li^+, by contrast, the class IIA ions had the opposite specificity, the Mg^{2+}, the ion of least radius, being by far the most active. Mn^{2+}, although having a greater effect even than Mg^{2+}, exhibited a concentration curve closely resembling that observed at 0.2 M NH_4^+.

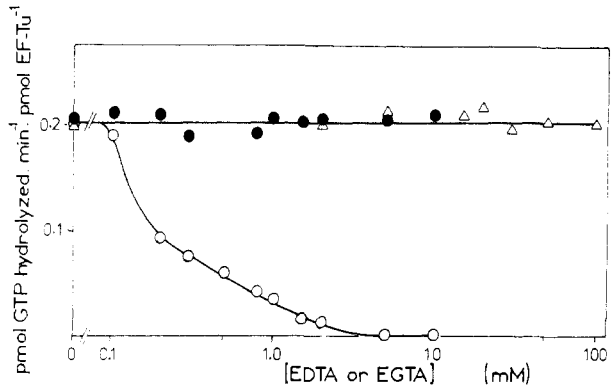

FIGURE 6: Effect of EDTA and EGTA on the EF-Tu·kirromycin GTPase activity in the absence of free Mg^{2+} ions. Reaction mixtures contained, in 75 μL, not more than 0.01 mM $MgCl_2$, imidazolium acetate, pH 7.5, 200 mM KCl, 20 pmol of EF-Tu, 50 μM kirromycin, 800 pmol of [γ-^{32}P]GTP (specific activity ~200 cpm/pmol), and Tris·H^+EDTA or Tris·H^+EGTA, pH 7.5, as indicated. (○) EDTA, (△) EGTA, and (●) EDTA plus 10 mM $MgCl_2$.

EF-Tu·Kirromycin GTPase in the Absence of Free Mg^{2+}. That EF-Tu·kirromycin displays a turnover GTPase activity in the absence of free Mg^{2+} was of great interest. We therefore explored this activity by titration against EDTA and EGTA whose chelating capacities for Mg^{2+} (log $K_{EDTA \cdot Mg^{2+}}$ = 8.9; log $K_{EGTA \cdot Mg^{2+}}$ = 5.4; Sillén, 1971; Williams, 1971) are quite different whereas they are comparable for the other class 2A metals. As illustrated in Figure 6, in the presence of 5 mM EDTA (open circles), all activity could be eliminated, EDTA itself showing no inhibition in the presence of an excess of Mg^{2+} (filled circles). EGTA (open triangles) was ineffective, up to 100 mM. At 0.1 mM EDTA, that is, at a concentration sufficient to chelate all free Mg^{2+} as well as that bound to the nucleotide, we could still observe more than 90% of the control activity. After inactivation of EF-Tu following incubation with 10 mM EDTA, addition of $MgCl_2$, in slight excess over EDTA, fully reactivated the GTPase activity of EF-Tu·kirromycin (not illustrated). These results suggest that a divalent cation, possibly Mg^{2+}, was still required for the GTPase activity of EF-Tu·kirromycin. The relatively large amount of EDTA necessary for complete inhibition implied that this cation must be very tightly bound to the EF-Tu·kirromycin complex and not only bound via substrate. However, measurements of the atomic absorption spectra (J. B. Crechet and A. Parmeggiani, unpublished results) have shown that neither Mg^{2+}, Ca^{2+}, Mn^{2+}, nor Zn^{2+} cations were present on an EF-Tu freed from GDP by incubation with EDTA and filtration on a Sephadex G-50 column (Fasano et al., 1978). EF-Tu treated in this way was still active in GTPase; its inactivation induced by adding 5 mM EDTA was fully reversed by an excess of Mg^{2+}, Ca^{2+}, or Mn^{2+}, but not by Zn^{2+}. Kirromycin preparations tested for Mg^{2+} and Mn^{2+} were found to be free from these metals.

Effect of pH and Mg^{2+} on the EF-Tu·Kirromycin GTPase. That enzymatic activity could be registered in the apparent absence of free or nucleotide-bound Mg^{2+} offered the possibility of checking the role of this ion in masking negative charges involved in the catalytic center and the extent to which Mg^{2+} could be functionally replaced by a monovalent cation, K^+. K^+ was chosen since it showed least influence on pH when present at high concentration. In Figure 7 are illustrated pH curves at two concentrations of KCl, 0.2 and 2 M, in the presence and absence of free Mg^{2+}. In the presence of Mg^{2+} (Figure 7B), curves at high and low [K^+] are similar in form with optima between pH 7.0 and 8.5. In the absence of free Mg^{2+} (Figure 7A), there is not only a marked shift of pH

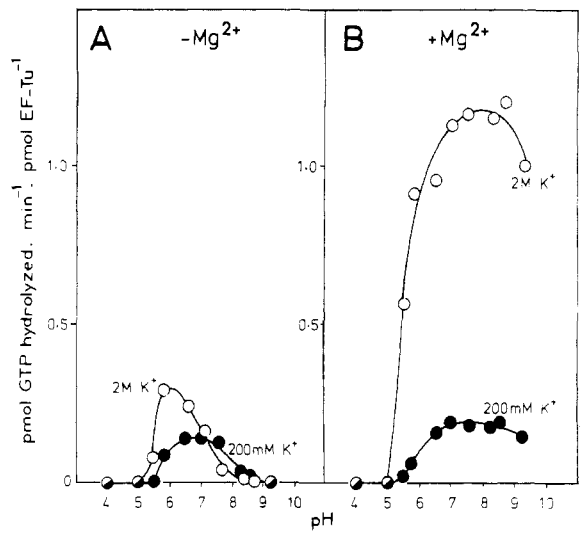

FIGURE 7: Effect of pH on the EF-Tu·kirromycin GTPase at different monovalent cation concentrations in the absence (A) or presence (B) of free Mg^{2+} ions. Reaction mixtures contained, in 75 μL, 5 mM $MgCl_2$, either 0.2 or 2 M KCl as indicated, 50 mM Tris-HCl of appropriate pH when measured under incubation conditions, 0.5 mM Tris·H^+EDTA, pH 7.5, 20 pmol of EF-Tu, 50 μM kirromycin, and 1 nmol of [γ-^{32}P]GTP (specific activity 200–250 cpm/pmol). (○) 2 M K^+; (●) 200 mM K^+.

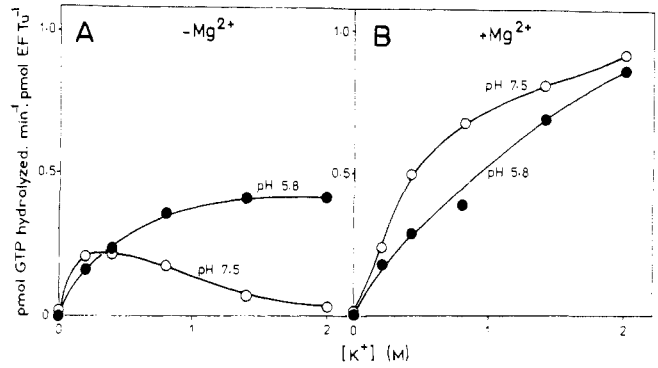

FIGURE 8: Effect of K^+ concentrations on the EF-Tu·kirromycin GTPase in different pH conditions in the absence (A) or presence (B) of free Mg^{2+} ions. Reaction conditions as in Figure 6. (●) pH 5.8; (○) pH 7.5.

optima to more acidic values but also a clear influence of K^+, high concentrations of which shift the pH optimum from 6.5–7.0 at 0.2 M K^+ to 5.8 at 2 M K^+. The converse experiments were also carried out (Figure 8) in which, in the presence and absence of Mg^{2+}, the effect of pH was assessed on the [K^+] optima for the EF-Tu·kirromycin GTPase. In the absence of Mg^{2+} (Figure 8A), the K^+ activity optimum was shifted to lower values by raising the pH from 5.8 to 7.5. A far less marked shift occurs in the presence of Mg^{2+} (Figure 8B). The significance of this interdependence between pH and [M^+] in the absence of Mg^{2+} is examined in the Discussion in the light of the polyelectrolyte theory of enzyme regulation (Douzou & Maurel, 1977; Maurel & Douzou, 1978). In the physiological system, in the absence of the antibiotic, no modulation by the pH of the [K^+] optimum for the EF-Tu GTPase has been observed (Sander & Crechet, 1978).

Influence of Divalent Cations on the Physiological EF-Tu GTPase in the Absence of Kirromycin. The presence of Mg^{2+} has been shown to affect the dependence of the EF-Tu GTPase on ribosome-bound mRNA in a system containing also EF-Ts and aa-tRNA (Sander, 1977). Therefore, in Figure 9, different divalent cations have been tested not only for their ability to stimulate the complete system but also for their

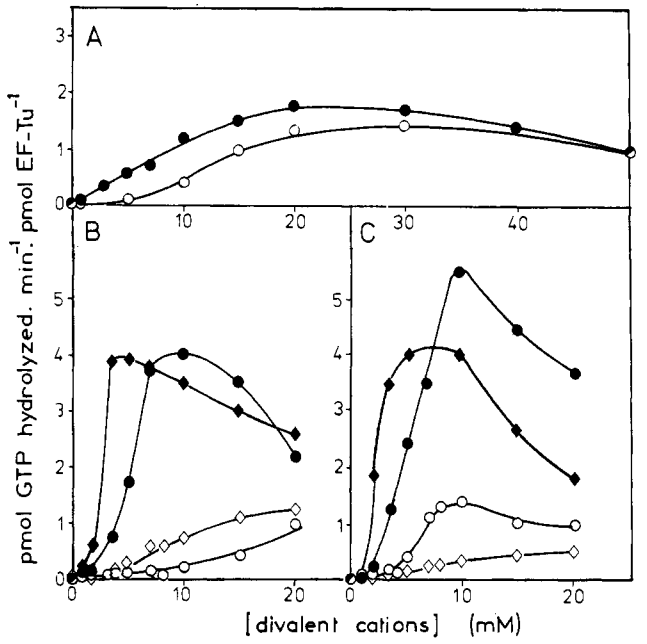

FIGURE 9: Influence of divalent cations in the physiological EF-Tu GTPase and its requirement for codon–anticodon interaction. Reaction mixtures contained, in 75 μL, 30 mM NH_4Cl, 0.5 mM Tris·H^+EDTA, pH 7.5, 50 mM imidazolium acetate, pH 7.5, 2 nmol of [γ-^{32}P]GTP (specific activity 100–150 cpm/pmol), 10 pmol of EF-Tu, 30 pmol of ET-Ts, 100 pmol of Phe-tRNA^{Phe}, and 20 pmol of ribosomes, with (filled symbols) or without (open symbols) 4 μg of poly(U). (A) Mg^{2+} (●, ○); (B) Ca^{2+} (♦, ◊); Ba^{2+} (●, ○); (C) Sr^{2+} (♦, ◊); Mn^{2+} (●, ○).

influence on the mRNA dependence of these EF-Tu GTPase activities.

In contrast to the kirromycin-containing system, no activity was registered in the absence of M^{2+} (0.5 mM EDTA). Mg^{2+} (Figure 9A) shows optimal stimulation at about 25 mM, though at this concentration there is almost no requirement for mRNA. At 5 mM Mg^{2+}, however, the GTPase activity, albeit lower, is almost entirely dependent on the presence of mRNA. The optimal concentrations for the other M^{2+}, in the presence of mRNA, are less than 10 mM, Ca^{2+} having the lowest at ~5 mM (Figure 9B), followed by Sr^{2+} (7 mM, Figure 9B), Ba^{2+} (~10 mM, Figure 9C), and Mn^{2+} (~10 mM, Figure 9C). There is also a large increase in total hydrolytic activity; at their respective concentration optima, Mn^{2+} is the most active cation (5.5 pmol of GTP hydrolyzed per pmol of EF-Tu in 1 min) and Mg^{2+} the least active (2 pmol of GTP hydrolyzed per pmol of EF-Tu in 1 min).

Discussion

The influence of M^+ and M^{2+} on the EF-Tu GTPase activity in various experimental conditions allows a number of conclusions to be drawn about the functional organization of the region of EF-Tu responsible for this activity as well as about the mechanism of action of the effectors aa-tRNA and ribosomes.

In the presence of kirromycin, M^+ are an absolute requirement whether with EF-Tu alone or plus aa-tRNA. In this system, two major responses to M^+ can be observed. At low concentrations (<0.5 M), there are specific effects of the different cations, leaving the K_m essentially unchanged whether or not aa-tRNA and/or ribosomes are present. This suggests more a long-range action via a conformational change of the enzyme–substrate complex than one involving the catalytic center of the reaction. That, except for NH_4^+, M^+ are unnecessary for maximum stimulation by both aa-tRNA and ribosomes probably depends on the presence of ions tightly bound to these effectors (Spitnik-Elson & Elson, 1976; Algranati & Goldenberg, 1977; Quigley et al., 1978). A different picture is obtained at $[M^+] > 0.5$ M when the GTPase activity of EF-Tu·kirromycin is proportional inversely to cation radius and directly to $[M^+]$, with the exception only of NH_4^+, whose effect differs from the small cations in that there is no increased hydrolysis above 1 M nor a change in K_m. The order of activity follows the increasing hydration energy, suggesting that the cations are binding to a site(s) of high anionic strength (Suelter, 1974); the corresponding changes in K_m indicate that this site is closely involved in the catalytic center for GTP hydrolysis. High concentrations increase the ability of the M^+ to substitute for aa-tRNA and ribosomes; effects of ionic type seem therefore to play an important role in the action of these two components. In calf adenylate deaminase (Setlow & Lowenstein, 1967), which also has the same unusual cation priority, it is similarly suggested that M^+ can substitute for an effector (adenosine triphosphate) or for a substrate (adenosine monophosphate) in inducing a shift from an inactive to an active conformation. The GTPAse activity of EF-Tu·kirromycin present at high $[M^+]$ curiously resembles the behavior of a halophilic enzyme. The relatively high content of polar residues found in EF-Tu (Arai et al., 1980), a feature also shared by halophilic enzymes, may be related to this property.

In the physiological system, just as for the stimulatory activity by aa-tRNA and ribosomes on the EF-Tu·kirromycin GTPase, M^+ are not necessary for optimal activity and inhibit already at low concentration. The highly specific series of interactions involving EF-Tu, ribosomes, and aa-tRNA thus appear more vulnerable to the intervention of strongly charged ions, whether or not kirromycin is present. Inhibition by increasing $[M^+]$ is also a feature of the EF-Tu GTPase activity found with aa-tRNA and ribosomes lacking mRNA at high $[Mg^{2+}]$ (Sander, 1977; this article; E. De Vendittis, personal communication) as well as of that found on addition only of ribosomes plus or minus methanol (Miller, 1972; Ballesta & Vazquez, 1973; Hamel & Nakamoto, 1972). That both K_m and the rate of GTP hydrolysis, in optimal conditions with or without kirromycin, tend toward similar values (~1 μM and 3 pmol of GTP hydrolyzed per pmol of EF-Tu in 1 min) as well as the preference in these conditions for the small cations Na^+ or Li^+ show the fundamental similarities of the two systems and implies that the effect induced by several large and complex macromolecules (ribosomes, aa-tRNA and EF-Ts) can be replaced by two types of very small ligands, kirromycin (M_r 796) and M^+, and by corollary that the physiological effectors function in respect to EF-Tu in a way similar to the cations and kirromycin. In line with these conclusions, by measuring the entropy of activation of the GTPase activity of EF-Tu·kirromycin, we have observed that increasing $[M^+]$ can partially substitute for the ribosomal effect (Bocchini et al., 1980). Recently, we have found that also in the absence of kirromycin, high concentrations of M^+ can activate the catalytic center of EF-Tu in the absence of ribosomes, aa-tRNA, and kirromycin. The properties of this hydrolysis will be reported elsewhere (Fasano et al., 1982).

An interesting finding of this study is that free Mg^{2+} ions are not required for GTPase activity in the presence of kirromycin. In other triphosphatase systems, a M^{2+} is thought to coordinate the substrate via the α- and β-phosphate groups, labilizing the γ-phosphate (Spiro, 1973). In studies on the binding of GDP to EF-Tu, it has been shown that a metal–substrate coordination takes place (Wilson & Cohn, 1977;

Wittinghofer & Lebermann, 1979; Goody & Lebermann, 1979); removal of the Mg^{2+} thus bound leads to a decreased affinity of EF-Tu for GDP by 3 orders of magnitude (Miller & Weisbach, 1970; Arai et al., 1974). Such a divalent metal coordination to the substrate is apparently not a prerequisite for the GTP hydrolysis of EF-Tu·kirromycin since under conditions which should remove all the free Mg^{2+} as well as that bound to the substrate, we could observe little inhibition of the reaction. With EF-Tu·kirromycin, other mechanisms, in which local charges or M^+ are implicated, may be involved. Suggestive in this sense is the presence of an interdependence between the pH and $[M^+]$ optima of the GTPase activity, a phenomenon which is only evident in the kirromycin system in the absence of free Mg^{2+} and which can be interpreted in the context of the polyelectrolyte theory of enzyme regulation (Maurel & Douzou, 1978). Starting from the consideration that electric charges of macromolecules may change the local ionic environment, such as the active center of an enzyme, these authors concluded that in such a situation the pH and salt optima of an enzymatic reaction should become interdependent. The marked acid shift of the EF-Tu·kirromycin GTPase activity in the absence of Mg^{2+}, which is augmented by higher $[M^+]$ would, according to this theory, imply a strong anionic region close to and intimately involved in the center of catalysis of EF-Tu. The Mg^{2+} ions would then have the role of modulators by screening this highly charged anionic field, the existence of which was already predicted from the effects of M^+ (see above). Since our results suggest that some tightly bound M^{2+} is responsible for the active conformation of the enzyme–substrate complex, at least in the kirromycin-dependent system, two roles should be assigned to M^{2+}, a low affinity role involved in nucleotide binding and regulation of the GTPase activity and a high affinity role essential for the catalysis and probably concerned with the structural integrity of EF-Tu, much as in the case of $tRNA^{Phe}$ (Quigley et al., 1978). Wittinghofer & Lebermann (1979) reported that Mg^{2+}, besides affecting the binding of GDP, also influences the tertiary structure of the factor. In the event that a M^{2+} might be tightly bound to EF-Tu, this would be neither Mg^{2+}, Ca^{2+}, nor Mn^{2+}, though these cations appear to be able to substitute for it (J. B. Crechet and A. Parmeggiani, unpublished results). Further studies are required to solve the several questions arising from the puzzling behavior of EDTA-treated EF-Tu in the presence of kirromycin.

The finding that for EF-Tu·kirromycin at high M^+ concentration there is first a considerable stimulation by Mg^{2+} and second a greater specificity for this cation, suggests that in these conditions there is a greater stringency in the M^{2+} requirement than in moderate M^+ conditions and that Mg^{2+} has here a very specific function. It may well be that in the presence of kirromycin the M^+, in inducing an active conformation of EF-Tu, also imposes greater constraints on the active site for catalysis and, hence, divalent cation requirement. Mn^{2+} is completely able to replace Mg^{2+} and, because of its low concentration optimum, evidently has a high affinity binding site, which differently to that of Mg^{2+} appears to be uninfluenced by $[M^+]$. A high affinity Mn^{2+} for EF-Tu·GTP has been reported by utilizing paramagnetic resonance techniques (Wilson & Cohn, 1977; Wittinghofer & Lebermann, 1979).

The role of ribosomes in stimulating the EF-Tu·kirromycin GTPase is especially interesting. At high $[M^+]$, ribosomes seem to act via a local ionic effect, e.g., an exchange of Mg^{2+} tightly bound to them, whereas at moderate $[M^+]$, there is an interaction between ribosomes and EF-Tu, wherein free Mg^{2+} has a considerable stimulatory role, implying that ribosomes display a more specific effect which cannot be substituted by Mg^{2+} alone.

The EF-Tu-dependent GTPase activity in the absence of kirromycin shows a complete dependence on M^{2+}, unlike the kirromycin system. Increasing $[M^{2+}]$ can progressively replace the requirement for mRNA [see also Sander (1977)], showing that the EF-Tu-dependent GTPase activity may occur uncoupled from proper codon–anticodon interaction which at lower M^{2+} levels is an absolute prerequisite (Thompson & Stone, 1977). This suggests an important role of M^{2+} in the recognition process. It is important to note that the mRNA-independent EF-Tu GTPase does not display the initial burst of GTP hydrolysis, which is strictly associated with the enzymatic binding of aa-tRNA to the ribosome, but an essentially linear kinetics (E. De Vendittis, private communication). Ca^{2+} would appear to have the highest efficiency in the physiological EF-Tu GTPase whereas Mg^{2+} displays the lowest one. Indeed, Ca^{2+} achieves optimum stimulation at low concentration and induces high GTPase activity, and at its optimum concentration, there is still almost a complete dependence on mRNA. The ability of Mg^{2+} to substitute for a macromolecular effector in the ribosomal reactions has also been seen for the binding of aa-tRNA to ribosome·mRNA, where increasing $[Mg^{2+}]$ can replace EF-Tu and, for the translocation reaction, where the function of elongation factor G can be substituted by choosing appropriate $[M^{2+}]$ (Belitsina & Spirin, 1979).

The results presented here illustrate the extreme complexity of the system coordinating the expression of the EF-Tu GTPase activity and emphasize the important role of local ionic effects in the function of the macromolecular ligands of EF-Tu.

Acknowledgments

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pH Dependence of Adenosine 5′-Triphosphate Synthesis and Hydrolysis Catalyzed by Reconstituted Chloroplast Coupling Factor†

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ABSTRACT: The purified ATP-synthesizing complex from chloroplasts has been reconstituted into phospholipid vesicles with bacteriorhodopsin by use of octyl glucoside. Phosphorylation rates up to 90 nmol of ATP (mg of protein)$^{-1}$ min^{-1} have been achieved. The dependence of the steady-state kinetic parameters on external and internal pH for both synthesis and hydrolysis was determined. The Michaelis constants are independent of the magnitude of the pH gradient at external pH values of 6.6 and 8.0. The dependence of the maximum velocity for ATP synthesis on the external pH is bell shaped at a constant pH gradient with a maximum at about pH 6.7. The variation of the maximum velocity with external pH is not dependent on the magnitude of the pH gradient. At external pH values of 6.6 and 8.0, the maximum velocity for ATP synthesis varies with approximately the 2.3 power of the internal hydrogen ion concentration. The maximum velocity for ATP hydrolysis also is dependent on the external pH, with a maximum at about pH 8.4; however, most of the ATPase activity is not coupled to the proton flux. Both Mg^{2+} and Mn^{2+} are good cofactors for ATP synthesis and hydrolysis whereas Ca^{2+} is completely ineffective for synthesis and only about 10% as effective as Mg^{2+} and Mn^{2+} for hydrolysis. The results obtained suggest that ATP synthesis or hydrolysis may be coupled to proton pumping indirectly, as, for example, by conformational changes.

The mechanism of ATP synthesis remains an unresolved question, despite the large amount of published work concerned with this problem (Boyer et al., 1977; Harris, 1978; Penefsky, 1979; Shavit, 1980; Fillingame, 1980). The chemiosmotic hypothesis (Boyer et al., 1977) forms a base for mechanistic considerations, but considerable controversy exists with regard to other aspects of the mechanism. We have embarked on a long-range study of the physical and catalytic properties of the purified dicyclohexylcarbodiimide-sensitive ATPase (DSA)[1] from chloroplasts (Pick & Racker, 1979; Baird et al.,

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[1] Abbreviations used: DSA, dicyclohexylcarbodiimide-sensitive ATP-synthesizing complex from chloroplasts; EDTA, ethylenediaminetetraacetic acid.

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