The influence of divalent metal ions on the intrinsic and kirromycin-stimulated GTPase activity of *Thermus thermophilus* EF-Tu has been investigated. The intrinsic GTPase activity under single turnover conditions varied according to the series: 

\[
\text{Mn}^{2+} (0.069 \text{ min}^{-1}) > \text{Mg}^{2+} (0.037 \text{ min}^{-1}) > \text{no Me}^{2+} (0.034 \text{ min}^{-1}) > \text{VO}^{2+} (0.014 \text{ min}^{-1})
\]

The kirromycin-stimulated activity showed a parallel variation. Under multiple turnover conditions (GTP/EF-Tu ratio of 10:1), 

\[
\text{Mg}^{2+}
\]

retarded the rate of hydrolysis in comparison to that in the absence of divalent metal ions, an effect ascribed to kinetics of nucleotide exchange. In the absence of added divalent metal ions, GDP and GTP were bound with equal affinity (\(K_d \approx 10^{-7} \text{ M}\)). In the presence of added divalent metal ions, GDP affinity increased by up to two orders of magnitude according to the series: 

\[
\text{no Me}^{2+} < \text{VO}^{2+} < \text{Mn}^{2+} - \text{Mg}^{2+}
\]

Estimates of equilibrium (dissociation) binding constants for GDP and GTP by EF-Tu on the basis of Scatchard plot analysis, together with thermodynamic data for hydrolysis of triphosphate nucleotides (Phillips, R. C., George, P., and Rutman, R. J. (1969) J. Biol. Chem. 244, 3330–3342), showed that divalent metal ions stabilize the EF-TuMe\(^{2+}\)-GDP complex over the protein-free Me\(^{2+}\)-GDP complex in solution, with the effect greatest in the presence of Mg\(^{2+}\) by \(-10 \text{ kJ/mol}\). These combined results show that Mg\(^{2+}\) is not a catalytically obligatory cofactor in intrinsic and kirromycin-stimulated GTPase action of EF-Tu in the absence of programmed ribosomes, which highlights the differential role of Mg\(^{2+}\) in EF-Tu function.

The GTPase superfamily of proteins, known more commonly as G-proteins, are ubiquitous in cellular systems and serve as key regulatory molecules catalyzing the hydrolysis of the \(\beta\,\gamma\)-phosphate bond in GTP (1–4). For most G-proteins this process yields a tightly bound enzyme-product complex requiring the action of a nucleotide exchange factor to kinetically facilitate exchange of the GTP for GDP in the active site. Nucleotide binding and hydrolysis are key to regulating the activity of these enzymes, and both of these steps occur in the presence of the divalent metal ion Mg\(^{2+}\) as the naturally occurring cofactor in the cell. However, in EF-Tu\(^1\) function it has not been possible hitherto to establish whether there is a differential role of the divalent metal ion in nucleotide hydrolysis as opposed to nucleotide binding.

In all G-proteins defined hitherto by x-ray crystallographic studies, the substrate analog guanosine 5\(^{\prime}\)-(\(\beta\,\gamma\)-imido)-triphosphate is bound as a complex with Mg\(^{2+}\) in which the divalent metal ion is coordinated to oxygen atoms from the \(\beta\)-phosphate and \(\gamma\)-phosphate groups, and the guanine and divalent metal ion binding sites appear to be tightly coupled (5, 6). For ras (7), for elongation factor Tu from both thermophilic bacteria (8–11) and *Escherichia coli* (12, 13), and for Ga-subunits of heterotrimeric G-proteins (14, 15), the dissociation constants for release of protein-bound nucleotide measured in the presence of Mg\(^{2+}\) indicate differential binding affinities for GDP and GTP by at least an order of magnitude. Binding affinities for both nucleotides are often measured kinetically by ligand displacement because G-proteins are less stable when prepared in their nucleotide-free form. Because nucleotide separation is necessary for complete removal of Mg\(^{2+}\) from the protein, the less stable, nucleotide-free form of G-proteins complicates efforts to determine whether the divalent metal ion has differential roles in nucleotide hydrolysis and nucleotide binding.

In contrast to the structural instability of elongation factor Tu of mesophiles such as *E. coli*, the homologous thermostable protein in its nucleotide-free form is less prone to inactivation, and comparison of the kinetics of nucleotide binding shows it to be identical to EF-Tu isolated as the GDP-bound complex from the cytosol (11, 16). This characteristic has allowed application of efficient biochemical purification methods for preparation of the nucleotide-free elongation factor from *Thermus thermophilus* (16, 17) and *Bacillus stearothermophilus* (18, 19) for x-ray structure analysis of the complex formed with an inhibitor analog of GTP in the active site (5, 6). Isolation of nucleotide-free elongation factor Tu (17–20) and p21\(^{ras}\) (7) has also allowed comparison of divalent metal ions on nucleotide binding and hydrolysis (8, 19, 21, 22) and the investigation of nucleotide-protein interactions by magnetic resonance methods (16, 21–26). In these studies, however, the question of whether the divalent metal ion is an absolute requirement for GTP hydrolysis has not been addressed.

In the present study we report results of kinetic and equilibrium binding studies to compare the influence of divalent metal ions on the intrinsic and kirromycin-stimulated GTPase activity of EF-Tu.*

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ity of EF-Tu of *T. thermophilus* in the absence of programmed ribosomes. We compared the influence of Mn$^{2+}$ and VO$^{2+}$ to that of Mg$^{2+}$ because they have been employed as paramagnetic probes of Mg$^{2+}$ function in thermostable EF-Tu (21, 23). Because we have been able to show that EF-Tu when depleted of nucleotide is not associated with protein-bound Mg$^{2+}$, we have been able to determine not only the influence of different divalent metal ions on the biological activity of EF-Tu without interference from adventitious Mg$^{2+}$ but also the intrinsic GTPase activity of the Mg$^{2+}$-free protein. The results lead to the conclusion that Mg$^{2+}$ is not a catalytically obligatory cofactor in the intrinsic and kirromycin-stimulated GTPase activity in the absence of programmed ribosomes. We also show that the binding of divalent metal ions provides the thermodynamic driving force for stabilization of the EF-Tu-Mg$^{2+}$-GDP complex, the effect being greatest with Mg$^{2+}$. The differential role of Mg$^{2+}$ in EF-Tu function is discussed further through analysis of thermodynamic relationships governing the hydrolysis of nucleoside 5'-triphosphates (27, 28) and the kinetic requirement for a nucleotide-exchange factor in the cell.

EXPERIMENTAL PROCEDURES

Materials

General—Guanosine nucleotides and BSA were obtained from Roche Molecular Biochemicals (Mannheim, Germany), [8-3H]GDP and [8-3H]GTP from Amersham Pharmacia Biotech, and [γ-32P]GTP from Hartmann Analytic (Braunschweig, Germany). Vanadyl sulfate hydrate was obtained from Aldrich (Milwaukee, WI) and Chelex 100 resin (dry mesh 50–100) was obtained from BioRad Laboratories (Hercules, CA). HEPES was purchased from Sigma (St. Louis, MO). All other reagents were of analytical reagent grade. Kirromycin was a gift from Gist-Brocades (Leiden, The Netherlands).

GTPase-free EF-Tu—EF-Tu was obtained as the EF-Tu-Mg$^{2+}$-GDP complex from *T. thermophilus* (17) or from the cell paste of *E. coli* (JM109) engineered with the tuf1 gene of *T. thermophilus* (HB8) (29). Nucleotide- and Mg$^{2+}$-free EF-Tu (EF-Tu) was isolated as described by Limmer et al. (16), as modified previously (21). For removal of GDP and Mg$^{2+}$, EF-Tu was diluted 1:6 into 5 m urea buffered to pH 5.85 with 0.01 M (NH$_4$)$_2$HPO$_4$. The resultant mixture with a protein concentration of ~1.5 × 10$^{-2}$ M was applied to a 1 × 52 cm column of CM-Sephadex CL-6B (Sigma) and eluted with the same buffer to remove GDP. A second buffer consisting of 0.01 M (NH$_4$)$_2$HPO$_4$ at pH 7.5, 5 m urea, and 0.05 M KCl was then applied to elute the protein. Renaturation of the protein was carried out by dialyzing against 0.05 M KCl and 0.15 m NH$_4$Cl or against 0.2 M KCl buffered with 0.05 m HEPES at pH 7.5. An Amicon YM-10 membrane (Amicon, Inc., Beverly, MA) extensively washed prior to use was employed to concentrate the protein solution. Whereas the original description for chromatography of EF-Tu and removal of Mg$^{2+}$ includes 0.01 M EDTA in the buffer (16), we found no difference in the results if EDTA was not present during denaturation and cation exchange chromatography of the protein. Therefore, to compare the influence of metal ions on nucleotide binding and GTPase activity, EDTA was not used during nucleotide removal to avoid the possible influence of low levels of adventitious EDTA. Use of glass containers also had to be avoided to prevent contamination with trace divalent metal ions. EF-Tu, prepared in this manner was stored at ~20 °C in the dialysis buffer containing 50% (v/v) glycerol.

Methods

Atomic Emission Spectroscopy—The Mg$^{2+}$-content of protein, nucleotide, kirromycin, and of all buffer solutions was determined by induc-tively coupled plasma atomic emission spectroscopy with a GBC Integra XMP spectrometer (BDC Scientific Equipment, Ltd., Dandenong, Victoria, Australia). The detection limit of this method is 1.0 × 10$^{-3}$ g Mg$^{2+}$ per liter or 4.0 × 10$^{-6}$ moles (g atoms) Mg$^{2+}$ per liter of solvent.

Determination of Nucleotide Binding Affinity—We observed previously that variability in the stoichiometry of nucleotide binding and biphasic character in Scatchard plots (30, 31) are essentially eliminated when BSA is added to the incubation mixture (21). Because nucleotide binding to BSA alone was not detected under these conditions, we concluded that BSA provided the role of a protectant neutral macro-

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2 Proteins are known to rapidly denature when used in dilute solutions and therefore, many enzyme preparations are provided in a mixture with added BSA as a source of neutral protein, e.g., Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) in *Molecular Cloning, A Laboratory Manual* (Nolan, C., ed) pp. 5.1-5.33, Cold Spring, Harbor Laboratory Press, NY.

3 We have confirmed by electron paramagnetic resonance experiments that VO$^{2+}$ is coordinated by NH$_2$ in the presence of NH$_4$Cl under the concentration conditions employed in these studies (A. Banerjee and M. W. Makinen, unpublished observations).
with the aid of the program GRAFT3.0 (Eritacus Software, Ltd., Staines, UK).

RESULTS

Removal of Mg$^{2+}$—In a variety of studies, the influence of divalent metal ions on the intrinsic, kirromycin-stimulated, and physiological GTPase activity of EF-Tu has been examined for EF-Tu of both E. coli (37, 38) and thermophilic (19, 20, 24) organisms. Also, the influence of monovalent and divalent metal ions on the intrinsic GTPase activity of p21$^{ras}$ has been compared with that of EF-Tu isolated from E. coli (39). In these studies, however, the question of whether Mg$^{2+}$ is a catalytically obligatory cofactor for GTP hydrolysis could not be addressed directly because the purified protein was associated with significant quantities of nucleotide and Mg$^{2+}$. An important motivation of the investigations reported here was to differentiate the role of Mg$^{2+}$ in GTP hydrolysis from that in nucleotide binding and to determine whether the divalent metal ion is an absolute requirement for GTPase action. For this reason it was necessary to ensure efficient removal of protein-bound Mg$^{2+}$.

We found that Mg$^{2+}$ could be consistently removed with high efficiency only under partial denaturation of EF-Tu in 5 M urea at pH 5.6 followed by cation exchange chromatography. As shown in Fig. 1, the concentration of Mg$^{2+}$ bound as the EF-Tu-Mg$^{2+}$-GDP complex shows a linear dependence on protein concentration. However, the amount of Mg$^{2+}$ associated with EF-Tu$_{N}$ when prepared as described under "Experimental Procedures," was consistently below the detection limit of the protein tryptophan fluorescence. Our result based on a nitrocellulose binding assay of 8$^{-2}$H)GTP is in good agreement with estimates of 1.8 $\times$ 10$^{-9}$ M made by Wagner et al. (10) and Arai et al. (9, 12), respectively, for the T. thermophilus protein. Furthermore, Wagner et al. (10) report a $K_{d}$ of 29 $\times$ 10$^{-9}$ M for the binding of GDP to EF-Tu$_{N}$ that was obtained by kinetic stopped-flow measurements of ligand displacement using protein tryptophan fluorescence. Our result based on a nitrocellulose binding assay of 8$^{-2}$H)GTP is in good agreement with the stopped-flow measurement of Wagner et al (10). On this basis we can conclude that very little GTP hydrolysis occurred under the experimental conditions for Scatchard analysis.

The influence of other divalent metal ions on nucleotide binding by T. thermophilus EF-Tu has not been previously investigated. Whereas Mg$^{2+}$ and Mn$^{2+}$ show equivalent effects in the binding of GDP to EF-Tu, as seen in Table I, the affinity for GDP in the presence of VO$^{2-}$ is decreased by a factor of 0.5. On the other hand, both VO$^{2-}$ and Mn$^{2+}$ facilitate tighter binding of GTP than does Mg$^{2+}$. It is of interest to note that the binding affinity is not only decreased by one to two orders of magnitude in the absence of added divalent metal ion but also both nucleotides are bound with equal affinity.

GTPase Activity—Fig. 2 illustrates progress curves for EF-Tu-catalyzed hydrolysis of GTP in the presence of Mg$^{2+}$, Mn$^{2+}$, and VO$^{2-}$ in the absence of added divalent metal ion under single turnover conditions. The activity in the presence of Mn$^{2+}$ is greater than in the presence of Mg$^{2+}$, which in turn is identical to the hydrolytic activity in the absence of added divalent metal ion. The intrinsic GTPase activity of EF-Tu$_{N}$ in the absence of divalent metal ion has not been previously reported, and the magnitude of the effect is comparable with that in the presence of Mg$^{2+}$. For these reasons we took special precautions to ensure that the protein and all reagents were exhaustively depleted of adventitious Mg$^{2+}$, as described under "Experimental Procedures."

### TABLE I

Comparison of stoichiometric binding coefficients and dissociation (equilibrium) binding constants of GDP and GTP to EF-Tu$_{N}$ in the presence or absence of added divalent metal ions determined by Scatchard plot analyses

| Metal ion | GDP | GTP |
|-----------|-----|-----|
| Mg$^{2+}$ | $3.7 \pm 1.3$ | $1.0 \pm 0.1$ |
| Mn$^{2+}$ | $3.2 \pm 1.3$ | $0.9 \pm 0.1$ |
| VO$^{2-}$ | $18 \pm 9$ | $0.9 \pm 0.2$ |

$a$ Results given are the average of at least three separate determinations.

$b$ Dissociation binding constants $K_{d}$ are given in units of 10$^{-9}$ M.

![Fig. 1. Mg$^{2+}$ content of EF-Tu determined by inductively coupled plasma atomic emission spectroscopy.](http://www.jbc.org/)

Unfattted EF-Tu-Mg$^{2+}$-GDP as purified from the cell (●); Mg$^{2+}$- and nucleotide-free EF-Tu (■).
Role of Mg$^{2+}$ in EF-Tu Action

FIG. 2. Hydrolysis of EF-Tu-bound GTP determined by release of [$^{32}$P]phosphate from [$^{32}$P]GTP catalyzed by EF-Tu, under single turnover conditions. The protein was added to the reaction mixture (0.05 M KCl buffered to pH 7.5 with 0.05 M HEPES) to a concentration of 1.0 × 10^{-3} M. The reaction in this case was monitored for incubation at 37 °C. The added metal ions Mn$^{2+}$ (○) and Mg$^{2+}$ (□) were added to 0.01 M concentration, whereas VO$^{2+}$ (□□) was added to a final concentration of 1.0 × 10^{-3} M. (○ ○ indicates no added divalent metal ion.) Values of $k_{cat}$, obtained by non-linear least-squares fit of the data to a single exponential function, are reported in Table II.

Whereas Scatchard analyses summarized in Table I showed that binding of both GDP and GTP in the presence of VO$^{2+}$ is of greater affinity than in the absence of added divalent metal ion, we see in Fig. 2 that hydrolysis of GTP in the presence of VO$^{2+}$ is slower than in the absence of divalent metal ions or in the presence of Mg$^{2+}$. Whereas we are not able to provide a complete explanation of this difference at present, the affinity of VO$^{2+}$ for inorganic triphosphate to form a 1:1 complex is at least 2 orders of magnitude greater than that of Mg$^{2+}$ (40). This effect is probably because of increased covalency in metal-ligand interactions, which may hinder hydrolysis of the triphosphate moiety in the protein-nucleotide complex. The apparent inhibitory effect of VO$^{2+}$ can be abolished by substitution of NH$_4^+$ for K$^+$ in the reaction mixture whereas the reaction in the presence of Mg$^{2+}$ or Mn$^{2+}$ is not influenced by addition of NH$_4^+$. Because VO$^{2+}$ can be coordinated by amines and other nitrogen-donor ligands (41–43), the high concentration of NH$_4^+$ is likely to cause coordination to VO$^{2+}$, converting the system to be equivalent to that in the absence of added divalent metal ion.4

Table II compares the results of our studies showing the influence of divalent metal ions under single turnover conditions for both intrinsic GTPase and kirromycin-stimulated GTPase activity in the absence of programmed ribosomes. For measurements of intrinsic GTPase activity, the value of $k_{cat}$ at 0.037 min$^{-1}$ in the presence of Mg$^{2+}$ is in good agreement with that of 0.042 min$^{-1}$ reported by Zeidler et al. (11). On the other hand, the value of $k_{cat}$ for GTP hydrolysis in the presence of Mn$^{2+}$ is 0.069 min$^{-1}$, greater than that observed in the presence of Mg$^{2+}$. The effect of Mn$^{2+}$ on accelerating hydrolysis of GTP over that observed with Mg$^{2+}$ has been observed also for the intrinsic GTPase activity of the ras proteins p21, Ran, and Rap1A (44). As seen in Table II, the kirromycin-stimulated GTPase activity in the absence of programmed ribosomes follows a similar dependence on added divalent metal ion to that observed for intrinsic GTPase activity.5

The hydrolysis of GTP catalyzed by EF-Tu, under multiple turnover conditions is illustrated in Fig. 3. It is seen that substrate turnover is faster in the presence of Mn$^{2+}$ than in the presence of Mg$^{2+}$. On the other hand, substrate turnover is slower in the presence of Mg$^{2+}$ than in its absence. This latter observation affirms that significant amounts of contaminant Mg$^{2+}$ could not have been present in the metal-free case under multiple turnover conditions because substrate turnover is faster in the absence of added divalent metal ion. Substrate hydrolysis under multiple turnover conditions occurs because of a combination of the intrinsic GTPase action of the protein together with nucleotide exchange. The different pattern of the influence of metal ions under multiple turnover conditions, therefore, must take into account the relative catalytic effects observed under single turnover conditions, as modulated by metal-dependent variations in the on- and off-rate constants for substrate binding and product release.

As evident in Table I, the affinity of EF-Tu, for GDP is identical in the presence of Mg$^{2+}$ and Mn$^{2+}$ whereas the affinity for GTP is slightly greater in the presence of Mn$^{2+}$. Therefore, because the rate of exchange of GDP by GTP must be essentially the same in the presence of Mn$^{2+}$ and Mg$^{2+}$, the enhanced rate of GTP hydrolysis with Mn$^{2+}$ may be because of either a decreased off-rate constant for GTP binding under multiple turnover conditions or a different involvement chemically of the metal ion in the reaction. Because the kinetics of product release do not influence the single turnover results, we attribute the enhanced intrinsic GTPase activity of the Mn$^{2+}$-enzyme to these same factors. Fig. 2 shows that the intrinsic GTPase activity of the enzyme in the absence of added divalent metal ion is unaltered from that in the presence of Mg$^{2+}$. The binding of GDP in the absence of divalent metal ion is significantly weaker than in the presence of Mg$^{2+}$ or Mn$^{2+}$, allowing even faster exchange of GDP by GTP under multiple turnover conditions. Because the intrinsic GTPase activity in the absence of added divalent metal ion is unaltered under single turnover conditions, despite a lower affinity for GTP, the hydrolytic activity in multiple turnover experiments must be considered as only apparently enhanced over that of the Mg$^{2+}$-enzyme because of faster nucleotide exchange.

**DISCUSSION**

The GTPase activity of EF-Tu of *T. thermophilus* depleted of bound nucleotide and divalent metal ion has not been previously investigated in the absence of added divalent metal ions. The main observation, as demonstrated through Figs. 2 and 3 and Table II, is that the intrinsic GTPase activity and the kirromycin-stimulated GTPase activity of *T. thermophilus*

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4 We have observed by Scatchard analyses that the binding of nucleotide by EF-Tu, in the presence of VO$^{2+}$ and 0.15 M NH$_4$Cl results in values of dissociation constants equivalent to those observed in the absence of added divalent metal ion (A. Banerjee and M. W. Makinen, unpublished observations).

5 Because it was likely that VO$^{2+}$ would have additional binding interactions with kirromycin, different from those of the other divalent metal ions studied, and because we had limited quantities of the antibiotic, we did not evaluate kirromycin-stimulated GTPase activity in the presence of VO$^{2+}$.
EF-Tu depleted of protein-bound Mg\(^{2+}\) is equivalent to that in the presence of added Mg\(^{2+}\). This result means that Mg\(^{2+}\) does not have a direct catalytic role in the intrinsic and kriromycin-stimulated hydrolysis of GTP catalyzed by EF-Tu in the absence of programmed ribosomes. Polypeptide chain elongation on the ribosome is estimated to proceed at a rate of \(\sim 600\) peptide bonds/ribosome/minute \((45)\) requiring hydrolysis of one GDP per elongation step. Because this is significantly greater than the value of \(k_{\text{cat}}\) of \(-0.037\) min\(^{-1}\) observed for intrinsic GTPase activity, as reported here and by others \((11, 46)\), the rate-limiting step cannot be the same as for the physiological reaction of EF-Tu-catalyzed GTP hydrolysis in the presence of programmed ribosomes. If the change in rate-limiting step in the presence of programmed ribosomes is a consequence of direct participation of Mg\(^{2+}\) in catalysis, structural relationships of the divalent metal ion and the triphosphate moiety of GTP in the active site of the EF-Tu-Mg\(^{2+}\)-GTP complex must differ significantly from those described heretofore \((5, 6)\). Thus, the results of our studies indicate not only that our understanding of the structural and chemical role of Mg\(^{2+}\) in the biological function of EF-Tu is incomplete but also that the physiological relevance of the x-ray structure of the EF-Tu-Mg\(^{2+}\)-guanosine 5’-(\(\beta,\gamma\)-imido)-triphosphate complex \((5, 6)\), prepared and crystallized in the absence of ribosomes through methods comparable with those applied here, is unclear.

The differential influence of divalent metal ions on GTPase activity and the nucleotide binding affinity can be analyzed on the basis of thermodynamic relationships in Scheme 1. For the closed system of reversible reactions constructed in Scheme 1 for hydrolysis of the free Me\(^{2+}\)-GTP complex and, correspondingly, of the EF-Tu-Me\(^{2+}\)-GTP complex, the reactions for GTP and GDP binding and release run along the vertical direction whereas the hydrolysis of free and EF-Tu-bound Me\(^{2+}\)-GTP are represented horizontally. The protein-bound and free states of the Me\(^{2+}\)-GTP and Me\(^{2+}\)-GDP complexes are coupled through equilibria for the binding and release of each Me\(^{2+}\)-nucleotide complex by EF-Tu. The free energy change associated with binding of each metal ion-nucleotide complex is estimated on the basis of the equilibrium (dissociation) constants in Table I. As the best approximation of the free energy change for hydrolysis of the free Me\(^{2+}\)-GTP complex, we apply the values determined through calorimetric and titration studies of George and co-workers \((27, 28)\) for adenosine 5’-triphosphate under conditions of pH, ionic strength, and Mg\(^{2+}\) concentration corresponding to those in our investigations. Because the closed series of linked equilibria in Scheme 1 requires that \(\Sigma \Delta G = 0\), the influence of divalent metal ions on the EF-Tu-catalyzed hydrolysis of the Me\(^{2+}\)-GTP complex with respect to the corresponding hydrolytic reaction of the free Me\(^{2+}\)-GTP complex in solution can be quantified. This analysis assumes that the free energy change for hydrolysis of each Me\(^{2+}\)-GTP complex in its free form can be approximated by that for Mg\(^{2+}\)-GTP. Whereas the analysis is rigorously applied in the case of Mg\(^{2+}\), we anticipate that the error attributed to this assumption for the other divalent metal ions, particularly Mn\(^{2+}\), is not significant.

With respect to the \(\Delta G^{\circ}\) of \(-34.9\) kJ/mol for hydrolysis of free Mg\(^{2+}\)-GTP in solution, the results in Table III show that the differential binding affinity of GDP and GTP by EF-Tu, in the presence of divalent metal ions results in an increasing thermodynamic driving force to stabilize the EF-Tu-Me\(^{2+}\)-GDP complex according to the series VO\(^{3+}\) \(<\) Mn\(^{2+}\) \(<\) Mg\(^{2+}\). Whereas Mg\(^{2+}\) retards hydrolysis of GTP in the absence of protein, a process that underlies the stability of nucleoside 5’-triphosphates in the cell, Mg\(^{2+}\) favors formation of the EF-Tu-Me\(^{2+}\)-GDP complex by \(-10\) kJ/mol over the free Mg\(^{2+}\)-GDP complex under comparable conditions of ionic strength, pH, and Mg\(^{2+}\) concentration. Furthermore, it is clear that the protein alone is not responsible for this action because EF-Tu is not associated with differential binding affinity for GTP and GDP in the absence of divalent metal ion. Also, the thermodynamic driving force of Mg\(^{2+}\) to stabilize the EF-Tu-Mg\(^{2+}\)-GDP complex is independent of the catalytic role that Mg\(^{2+}\) may have in the physiological GTPase activity of EF-Tu in the presence of programmed ribosomes.

The analysis through Table III and Scheme 1 affirms the generally held notion that the role of Mg\(^{2+}\) is to stabilize the
EF-Tu-Mg\(^{2+}\)-GDP complex. This protein-nucleotide complex in turn serves as a pivot point in the regulation of the activity cycle of EF-Tu. Because the cellular content of GTP is at least 7-fold greater than that of GDP (47), EF-Tu-bound GTP upon hydrolysis in the cell would lead to saturation of the protein as the tightly bound EF-Tu-Mg\(^{2+}\)-GDP complex simply because of the ubiquitous presence of Mg\(^{2+}\). Thus, the thermodynamic role of Mg\(^{2+}\) in EF-Tu catalyzed GTP hydrolysis requires the presence of a nucleotide exchange factor in the cell to kinetically facilitate replacement of GDP by GTP. Comparison of the x-ray structures of the GTP (5, 6) and GDP (48, 49) bound forms of EF-Tu shows significant structural changes involving not only local changes in the metal ion and nucleotide binding sites but also global changes involving shifts of domains II and III. The binding surfaces on EF-Tu involved in binding to EF-Ts are located in the G domain and domain III, and their arrangement remains similar upon formation of the EF-Tu-EF-Ts complex to that in the EF-Tu-Mg\(^{2+}\)-GDP complex. It is thought that this structural relationship underlies the kinetic preference of EF-Ts to bind to the GDP complex of EF-Tu over the GTP complex (50). On this basis, it is likely that the role of Mg\(^{2+}\) in stabilizing the GDP complex facilitates recognition of the EF-Tu-Mg\(^{2+}\)-GDP complex by EF-Ts. It is of interest to note that binding of T. thermophilus EF-Tu by the nucleotide exchange factor EF-Ts results in insertion of the Phe-82 residue of EF-Ts into the hydrophobic pocket of EF-Tu formed by His-85, Leu-122, and His-119, disturbing the Mg\(^{2+}\)-phosphate portion of the active site (50), and the occupancy of Mg\(^{2+}\) in the binding pocket, as established by x-ray studies of the Tu complex (50, 51), is abolished. Thus, in the biological action of EF-Tu, Mg\(^{2+}\) binding stabilizes the conformation of the EF-Tu-Mg\(^{2+}\)-GDP complex to facilitate recognition by EF-Ts, but destabilization of Mg\(^{2+}\) binding in the active site of Tu is likely to be critical to initiate nucleotide exchange.

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