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The Structure of the EF-Tu · GDP · Me^{2+} Complex

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The structure of the MgGDP complex at the active site of elongation factor (EF-Tu) has been investigated by using phosphorothioate analogs of GDP in the absence and presence of various metal ions, electron paramagnetic resonance (EPR) and nuclear magnetic resonance (NMR) measurements. The high stereoselectivity of EF-Tu for the diastereomers of guanosine 5′-*O*-(1-thiodiphosphate) (GDP[αS]) is independent of the nature of the metal ion and is caused by the interaction of the protein with the α-phosphate of GDP. By using GDP analogs where the oxygens at either the α-phosphate or the β-phosphate have been selectively labelled with ^{17}O and measuring their effect on the EPR spectrum of EF-Tu-bound manganese we are able to show that only the β-phosphate of GDP is coordinated to the metal ion in the EF-Tu · Me^{2+} · GDP complex. ^{31}P-NMR studies on GDP and guanosine 5′-*O*-(2-thiodiphosphate) (GDP[βS]) bound to EF-Tu indicate that in the EF-Tu · Me^{2+} · GDP complex Mg^{2+} interacts more strongly with the β-phosphate than with the α-phosphate. Together with binding studies using GDP[βS] our NMR results also indicate that the protein is complexed to the β-phosphorous of GDP via two oxygens.

Pure diastereomeric thiophosphate analogs of ADP and ATP have been prepared [1] and it has been found that enzymes which interact with these analogs usually have a pronounced specificity for one diastereomer [2,3]. Recently Goody and Leberman [4] have prepared GDP[βS] and the diastereoisomers of GDP[αS] and studied their interaction with polypeptide elongation factor (EF-Tu) from *Escherichia coli*. From their results they concluded that a α,β-bidentate MgGDP complex is the substrate recognized by EF-Tu and that the interaction of GDP with the enzyme at the β-phosphate might involve two non-bridging oxygens. Here we describe a study of the affinity of these thiophosphate analogs to EF-Tu from *Bacillus stearothermophilus* which, because of its inherent stability in the nucleotide and metal-free form ($EF\text{-}Tu_f$ [5]), is better suited than EF-Tu from *E. coli* for these measurements. The results obtained in the absence and presence of various metal ions render the first conclusion of Goody and Leberman unlikely, but support the second one. We conclude from these studies that Mg · GDP is bound as its β-monodentate complex.

By making use of the spectrum of manganese ion bound to EF-Tu in the presence of ^{17}O-enriched analogs of GDP, we confirm that our model is correct and that in the active site of EF-Tu there is an interaction of the GDP with Mn^{2+} only via the β-phosphate group. Further confirmation of our model is provided by ^{31}P-NMR studies on complexes between EF-Tu, nucleotides, and magnesium ions.

MATERIALS AND METHODS

Unless otherwise stated, all materials were commercially available. The GDP thiophosphate analogs GDP[αS] isomers A and B and GDP[βS] were prepared as described [4]. GDP was obtained from Pharma Waldhof and [^3H]GDP (569 GBq/mmol) from Amersham Buchler. $MgCl_2$, $Co(NO_3)_2$ and $Cd(OAc)_2$ were analytical grade reagents. EF-Tu · GDP from *Bacillus stearothermophilus* was prepared as described by Leberman et al. [6] and was converted to nucleotide-free and metal-free $EF\text{-}Tu_f$ as described [5]. The relative affinities of the thiophosphate analogs for EF-Tu in the presence of metal ions were measured by competition with [^3H]GDP as described [4]. EF-Tu · GDP was used in the presence of Mg^{2+} and $EF\text{-}Tu_f$ in the measurements with Co^{2+} and Cd^{2+}. Standard buffer for the inhibition experiments was 50 mM sodium 1,4-piperazineethanesulfonate, pH 6.8, and contained 1 mM Cd^{2+} or Co^{2+}, or 5 mM Mg^{2+} and either 1 mM dithiothreitol for the Mg^{2+} measurements of 1 mM ascorbic acid in the Co^{2+} and Cd^{2+} measurements. In the equilibrium dialysis measurements, this buffer in the presence of 0.1 mM EDTA was used. The three different batches of EF-Tu produced and used in the magnetic resonance experiments contained, for reasons unknown to us and contrary to earlier reports, varying amounts of EF-Tu · GDP. This was determined by analyzing the GDP binding ability after 15 s and after 90 min, the difference between these figures representing the amount of EF-Tu · GDP which exchanges slowly with external [^3H]GDP. Thus 20–25% of the total binding activity was found to be due to EF-Tu · GDP. $EF\text{-}Tu_f$ in standard Epps buffer [7] which had been passed twice through a chelating resin (Chelex 100) was concentrated in an Amicon B15 concentration cell to a concentration of about 1 mM and used immediately. $MnCl_2$ was added from a 50 mM stock solution in water to a final concentration as indicated.

Synthesis of ^{17}O-Enriched Nucleotides

[α-$^{17}O_3$]GDP. [$^{17}O_3$]GMP was prepared starting from $H_2{}^{17}O$, PCl_5 and guanosine without intermediate isolation of $P^{17}OCl_3$. This method is described in detail elsewhere [8]. [α-$^{17}O_3$]GDP was obtained by phosphorylation of [$^{17}O_3$]GMP in the following manner. 5 µl of guanosine monophosphate

Abbreviations. EF-Tu, polypeptide elongation factor Tu; GDP[αS], guanosine 5′-*O*-(1-thiodiphosphate); GDP[βS], guanosine 5′-*O*-(2-thiodiphosphate); EPR, electron paramagnetic resonance; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Epps, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid.

kinase (2 mg/ml; Boehringer, Mannheim) was added to a reaction mixture containing [$^{17}O_3$]GMP (15.4 μmol), ATP (66 μmol), $MgCl_2$ (5 mM) and Tris · HCl pH 8.0 (50 mM). After allowing to stand overnight at 4 °C, the mixture was separated by ion-exchange chromatography on DEAE-Sephadex A25, using a gradient of 0.2 – 0.4 M triethylammonium bicarbonate at 4 °C. [α-$^{17}O_3$]GDP was eluted ahead of ATP in a yield of about 65% based on [$^{17}O_3$]GMP. This procedure could be scaled up significantly without modification. When incomplete separation of [α-$^{17}O_3$]GDP from ATP was obtained in largescale preparations, the impure [α-$^{17}O_3$]GDP was incubated with myosin subfragment-1 and pyrophosphatase (to remove pyrophosphate detected by ^{31}P-NMR) in the presence of Mg^{2+} before rechromatography under similar conditions. The product was characterized by its ^{31}P-NMR spectrum; from the loss of the peak height of the signals of the phosphorous atom due to the quadrupole moment of ^{17}O it was estimated that the enrichment with ^{17}O at the α-phosphate was about 40 – 50% [9].

[β-$^{17}O_4$]GDP. K_2HPO_4 enriched with ^{17}O was prepared as previously described [10]. 0.5 mmol of this was used in the phosphorylation of GMP by the method described by Michelson [11]. A 1.25 molar excess of activated GMP over ^{17}O-enriched phosphate was used. After separation of the products by chromatography on DEAE-Sephadex, [β-$^{17}O_4$]-GDP was obtained in about 35% yield based on [^{17}O]phosphate. Again, ^{31}P-NMR was used to check the purity of the product, and the enrichment with ^{17}O on the β-phosphate was estimated to be about 40 – 50%.

EPR Measurements

The EPR measurements were performed with a Varian E-line spectrometer operating at 9.4 GHz and a Bruker BER-420 spectrometer operating at 33.9 GHz. For the X-band measurements at 9.4 GHz a time-averaging computer was added to improve the signal-to-noise ratio and to subtract the broad background signal of the sample-free cavity. The sample temperature was stabilized to 277 ± 0.5 K by using a nitrogen gas flow system. Samples were contained in calibrated quartz glass capillary tubes. The error given in Table 1 corresponds to the 95% confidence level obtained by applying the *t*-test on the experimental data.

NMR Measurements

NMR spectra were recorded on a commercial Bruker HX360 spectrometer working with a ^{31}P frequency of 145.7 MHz. The samples contained approximately 10% ^2H_2O to provide a signal for the lock unit of the spectrometer. All spectra were recorded at 15 °C. Chemical shifts are expressed with respect to 85% H_3PO_4 as external reference and given with increasing numbers in the direction of decreasing field.

RESULTS

Fig. 1 shows the results obtained when the EF-Tu · [^{3}H]-GDP complex was incubated in the presence of varying concentrations of GDP and the thiophosphate analogs GDP[αS] isomer A and B and GDP[βS]. Estimates of the ratios of the binding constants of analogs to those of GDP were obtained from plots of [EF-Tu · GDPanalog]/[EF-Tu · GDP] against [GDPanalog]/[GDP] as described [4]. The values obtained

Table 1. *Spectroscopic parameters for various EF-Tu · Mn^{2+} complexes at 9.4 GHz*
The mean line widths given are the average of all six hyperfine lines. The mean amplitudes are the average of all six hyperfine lines quoted relative to the EPR signal of an equal amount of free manganese where the mean amplitude per mol manganese at 277 K is defined arbitrarily as 1

Sample	Mean linewidth	mean amplitude
	mT	
Free Mn^{2+}	3.67	1.00
EF-Tu · Mn^{2+}	4.13	0.19
EF-Tu · Mn^{2+} · GDP	5.08	0.15
EF-Tu · Mn^{2+} · GTP	5.00	0.14

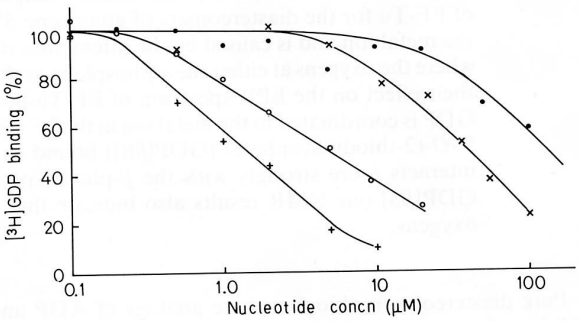

Fig. 1. *Inhibition of [^{3}H]GDP binding to EF-Tu.* Samples (250 μl) containing 0.1 μM EF-Tu · GDP, 1.25 μM [^{3}H]GDP (56.9 GBq/mmol) and various concentrations of GDP or analog in standard buffer with 5 mM Mg^{2+} were incubated at room temperature for 90 min. Concentrations of (+) GDP, (○) GDP[αS] isomer A, (×) GDP[βS] and (●) GDP[αS] isomer B are as indicated. The samples were filtered through nitrocellulose filters and counted as described [4]. 100% binding corresponds to 22000 counts/min

were 0.23, 0.041 and 0.0083 for GDP[αS] isomer A, GDP[βS] and GDP[αS] isomer B and thus very closely resemble the respective affinities of these analogs to EF-Tu from *Escherichia coli*. The ratio of affinities of isomer A and B (A/B ratio) is 27 as compared to 23 for *E. coli*, and the affinity of GDP[βS] relative to GDP is 0.041 versus 0.021 for the EF-Tu from the two bacteria. Small variations in these numbers do not necessarily indicate a different active site structure but are more probably caused by slight impurities in the different batch preparations of analog.

It has been postulated [4] that EF-Tu recognizes the α,β-bidentate MgGDP complex. By using the diastereomers of GDP[αS] and replacing Mg^{2+} by other divalent metal ions which have a higher tendency to complex with sulfur, it should be possible to test this model as other investigators have done for various ADP and ATP binding enzymes [2, 3] (see also Fig. 2). Fig. 3 shows the inhibition of [^{3}H]GDP binding by GDP and its thiophosphate analogs in the presence of 1 mM Cd^{2+}. It should be noted that ascorbic acid was used as an antioxidant instead of dithiothreitol to protect the protein since dithiothreitol forms a precipitate with Cd^{2+} or Co^{2+}. Evaluation of the data in Fig. 3 leads to an order of affinities which is GDP[αS] isomer A > GDP > GDP[βS] > GDP[αS] isomer B with relative binding constants of approximately 2.94 : 1 : 0.40 : 0.19. This shows that the relative order of affinities of the diastereomers A and B of GDP[αS] is not reversed and that the A/B ratio is around 15. Fig. 3 also

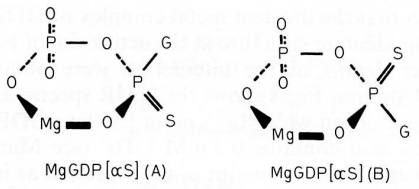

Fig. 2. *Structures of the α,β-bidentate Mg^{2+} complexes of the diastereomers of GDP[αS].* On substituting Cd^{2+}, which binds preferentially to sulfur, for Mg^{2+}, a structure analogous to MgGDP[αS] (A) should be formed by CdGDP[αS] (B), and vice versa, so that if the bidentate complexes are recognized by EF-Tu, a reversal of specificity for the diastereomers should then be observed

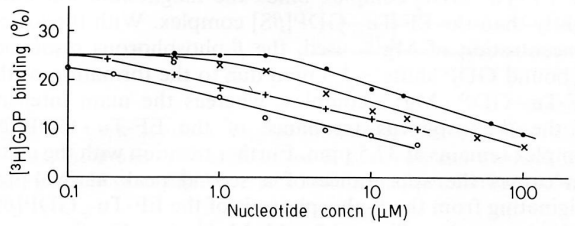

Fig. 3. *Inhibition of [^{3}H]GDP binding to EF-Tu in the presence of Cd^{2+}.* 0.19 μM EF-Tu_f was incubated in standard buffer with 1 mM Cd^{2+} in the presence of [^{3}H]GDP and analogs as described in Fig. 1. 100% binding, which was obtained with 5 mM Mg^{2+} under identical conditions, corresponds to 45000 counts/min

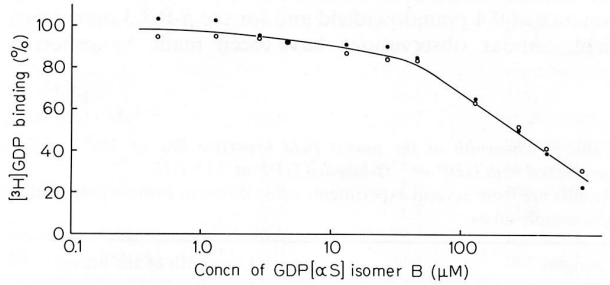

Fig. 4. *Comparison of the effect of Mg^{2+} and Co^{2+} on the inhibition of GDP binding by GDP[αS] isomer B.* Inhibition of the binding of 4.2 μM [^{3}H]GDP to 0.35 μM EF-Tu_f in the presence of 1 mM Co^{2+} (○) or 5 mM Mg^{2+} (●) with concentrations of analog as indicated

shows that the selectivity of EF-Tu in the presence of Cd^{2+} is altered in that GDP[αS] isomer A is now a better substrate than GDP and that the affinities of all three thiophosphate analogs relative to GDP are much higher than with Mg^{2+}. It is also apparent that the GDP binding activity drops to 25% of its control value. This inactivation of EF-Tu by Cd^{2+} is a very specific effect and will be described in a separate report.

We showed earlier [5] that Co^{2+} appears to be a good analog of Mg^{2+} in the GDP binding site of EF-Tu since it can stimulate GDP binding to EF-Tu in a manner similar to Mg^{2+} and Mn^{2+}. Since the use of Cd^{2+}, but not of Co^{2+}, leads to a partial inactivation of the elongation factor and since Co^{2+} has an affinity for sulfur which is intermediate between those of Mg^{2+} and Cd^{2+}, we measured the affinities of the diastereoisomers of GDP[αS] in the presence of Co^{2+} and compared them with the results obtained with Mg^{2+}. We find that the inhibition curves are identical for Mg^{2+} and Co^{2+} as is shown for GDP[αS] isomer B in Fig. 4 (results for other

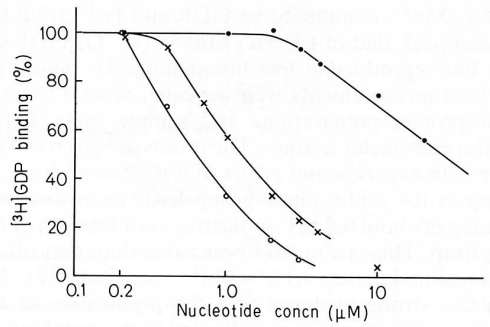

Fig. 5. *Inhibition of [^{3}H]GDP binding in the absence of metal ions.* 0.2 μM EF-Tu_f was dialyzed against 0.2 μM [^{3}H]GDP (569 GBq/mmol) in the presence of different concentrations of analog in 200-μl dialysis cells as described [7]. After 20 h at 4 °C 50-μl aliquots from both sides of the dialysis chamber were counted and the difference between the two sides is taken as the amount of [^{3}H]GDP bound to EF-Tu. 100% binding corresponds to 60000 counts/min. (●) GDP[αS] isomer B; (×) GDP[βS]; (○) GDP[αS] isomer A

analogs not shown) and thus the relative affinities of GDP[αS] isomer A and B and GDP[βS] are not altered.

Since the nature of the metal ion does not influence the A/B ratio, we were interested in measuring the relative affinities of the analogs in the absence of divalent metal ions. The affinity of GDP for EF-Tu in the absence of metal ions has been determined to be 10^7 M^{-1} by equilibrium dialysis [7] and here we determined its inhibition by the GDP analogs using the same method, as shown in Fig. 5. The A/B ratio is 42, which is reasonably close to the value obtained in the presence of Mg^{2+}, Co^{2+} and Cd^{2+} and the affinities of GDP[αS] isomers A and B relative to GDP are very similar for the absence and presence of Mg^{2+}. There is, however, a significant difference in the relative affinity of GDP[βS] which has an affinity of 0.16 relative to GDP as compared to 0.041 in the presence of Mg^{2+}.

In order to define the nature of protein-nucleotide-metal ion interaction further magnetic resonance measurements were also performed. Mn^{2+} has been found to be a good analog of Mg^{2+} at the active site of EF-Tu and to bind with an affinity constant of 10^5 M^{-1} [7]. The EPR spectrum of EF-Tu-bound Mn^{2+} with the characteristic six-line pattern is shown in Fig. 6. No additional EPR signal has been found outside the region centered around $g = 2$. On addition of GDP or GTP to the EF-Tu · Mn^{2+} complex, there is a significant broadening of the EPR spectrum (Fig. 5 and Table 1) but the principal spectral features remain unchanged.

We applied the method used by Reed and Leyh to define the ligands of Mn^{2+} in an ADP–creatine-kinase complex [12] to provide direct proof of this interaction and to define the structure of the Me · GDP complex at the active site of EF-Tu. [α-$^{17}O_3$]GDP and [β-$^{17}O_4$]GDP, in which the oxygens attached to either the α or β-phosphorous atoms were enriched to the extent of 40–50% with ^{17}O, were prepared and used in experiments with EF-Tu · Mn^{2+}. If a ^{17}O-enriched phosphate group is coordinated to the manganese ion the EPR spectrum should be broadened due to the unresolved splitting caused by the superhyperfine interaction between Mn^{2+} and the ^{17}O nucleus ($I = 5/2$) [12, 13]. Fig. 7 shows the two low-field lines of the EF-Tu · GDP · Mn^{2+} spectrum at 33.9 GHz. As expected, the line width at the higher frequency is remarkably reduced, by a factor of about 4, allowing more precise measurements of possible line broadening. Whereas the spectra

of EF-Tu · Mn^{2+} complexed to GDP and [α-$^{17}O_3$]GDP are almost identical, that of EF-Tu · Mn^{2+} · [β-$^{17}O_4$]GDP shows a small but reproducible line broadening. To quantify this effect, these measurements were repeated several times with different protein preparations and sample tubes but with identical spectrometer settings. The results are given in Table 2 together with experimental errors at a 95% confidence level. Labelling of the β-phosphate group leads to an overall line broadening of about 0.2 mT, indicating coordination of Mn^{2+} to this group. This is a much lower value than that observed in the creatine-kinase−ADP−Mn^{2+} complex [12], but is nevertheless strong evidence that the β-phosphte of GDP, but not the α-phosphate, is coordinated to the metal ion at the active site of EF-Tu. This confirms the result recently obtained by Eccleston et al. [14], of which we became aware in the final stages of this work, using the Mn^{2+} EPR method with EF-Tu from *E. coli*. These data, taken together with the results obtained using the thiophosphate analogs of GDP, allow the conclusion that the divalent metal complex of GDP is bound as a β-monodentate structure at the active site of EF-Tu.

Further details of the interaction were obtained from ^{31}P-NMR studies. Fig. 8 shows the NMR spectra as recorded during the titration with Mg^{2+} of an EF-Tu · GDP[βS] complex which also contains 0.3 mM GDP (see Materials and Methods) and a small amount of GTP present as impurity in the GDP[βS] preparation. Although not all nucleotide was bound to the protein, a distinction of the unbound form from the bound form of the nucleotides GDP[βS] and GDP was clearly possible, indicating the prevalence of slow exchange conditions between free and bound states. ($\tau_{ex}^{-1} < 100\ s^{-1}$ for the metal-free solution.)

As is evident from the spectrum taken at 1.4 mM Mg^{2+}, the EF-Tu · GDP complex binds the magnesium ion more tightly than the EF-Tu · GDP[βS] complex. With the lowest concentration of Mg^{2+} used, the β-phosphorous resonance of bound GDP shifts −1.5 ppm due to the formation of the EF-Tu · GDP · Mg^{2+} complex, whereas the main intensity of the β-phosphorus resonance of the EF-Tu · GDP[βS] complex remains at 37.5 ppm. Further titration with the metal ion causes the appearance of a second peak at 39.4 ppm originating from the β-phosphorous of the EF-Tu · GDP[βS] · Mg^{2+} complex. Since 4.2 mM Mg^{2+} (see Fig. 8) gives a 50% conversion to this complex we can estimate the binding constant of Mg^{2+} to be about $3 \times 10^2\ M^{-1}$, or a factor 10^3 lower than for the EF-Tu · GDP complex [7].

Table 3 shows a compilation of chemical shift data derived from these experiments. The following points can be made. (a) Binding of GDP to EF-Tu causes a shift of the α-P resonance of 0.4 ppm downfield and for the β-P 3.3 ppm downfield. Similar observations have been made by other in-

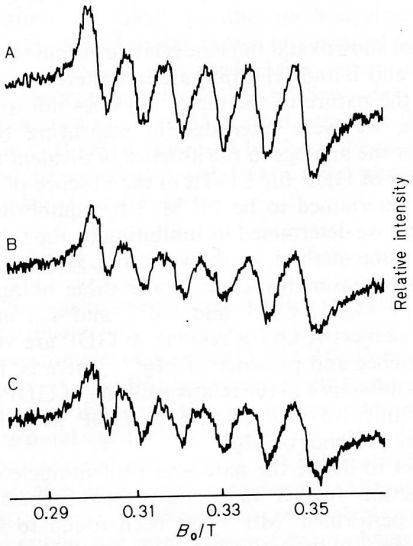

Fig. 6. *X-band spectra of Mn^{2+}-EF-Tu complexes.* Measurements were performed as described in Materials and Methods under identical spectrometer settings. The solution contained, besides standard buffer, the following components: (A) 200 μM Mn^{2+}, 758 μM EF-Tu. (B) 182 μM Mn^{2+}, 182 μM GDP, 689 μM EF-Tu. (C) 182 μM Mn^{2+}, 182 μM GTP and 689 μM EF-Tu

Table 2. *Linewidth of the lowest field hyperfine line of Mn^{2+} · EF-Tu complexed with GDP or ^{17}O-labelled GDP at 33.9 GHz*
Results are from several experiments using different protein preparations and sample tubes

Complex	Linewidth of the lowest hyperfine line
	mT
EF-Tu · Mn^{2+} · GDP	1.30 ± 0.02
EF-Tu · Mn^{2+} · [α-$^{17}O_3$]GDP	1.32 ± 0.02
EF-Tu · Mn^{2+} · [β-$^{17}O_4$]GDP	1.49 ± 0.02

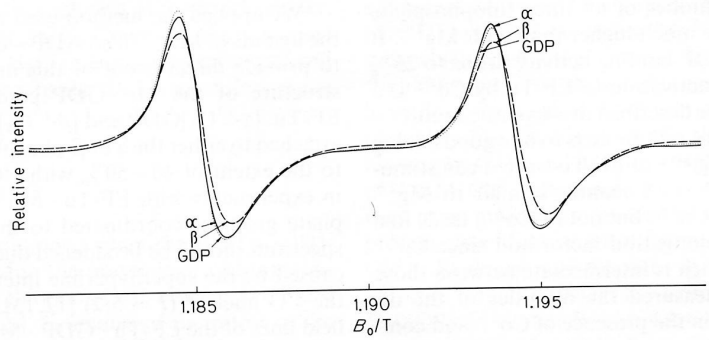

Fig. 7. *Q-band spectra of Mn^{2+}-EF-Tu complexed with GDP and ^{17}O-labelled nucleotides.* Measurements were performed as described in Materials and Methods. Only the two lowest field hyperfine lines are shown. The solution contained, in addition to standard buffer, 955 μM EF-Tu, 890 μM Mn^{2+} and 910 μM each of GDP (· · · ·), [α-$^{17}O_3$]GDP (——) or [β-$^{17}O_4$]GDP (– – –)

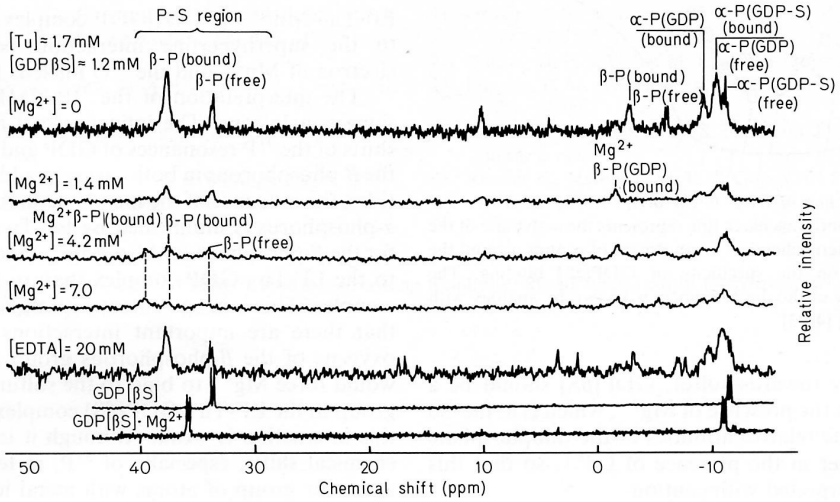

Fig. 8. *^{31}P-NMR spectra showing the effect of Mg^{2+} on the EF-Tu · GDP[βS] complex.* The solution contained 1.7 mM EF-Tu, 1.4 mM GDP[βS] and residual GDP and GTP as described in Materials and Methods with various amounts of $MgCl_2$ as indicated. For comparison, spectra of free GDP[βS] and of its Mg^{2+} complex have been added. Buffer was 50 mM Hepes · Na^+ at pH 7.1, 1 mM dithiothreitol

Table 3. *Chemical shift data for the α and β-phosphate resonances of various GDP and GDP[βS] complexes*

Complex	α-Phosphate	β-Phosphate	Reference
	ppm		
GDP	−10.3	− 6.1	[25]
GDP · Mg^{2+}	− 9.5	− 5.4	P. Roesch, unpublished
GDP[βS]	−11.1	33.6	[25]
GDP[βS] · Mg^{2+}	−10.6	35.7	P. Roesch, unpublished
EF-Tu · GDP	− 9.4	− 2.8	this work
EF-Tu · GDP · Mg^{2+}	− 8.9	− 1.5	this work
	− 9.6	− 1.7	[19]
EF-Tu · GDP[βS]	−10.5	37.5	this work
EF-Tu · GDP[βS] · Mg^{2+}	−10.7	39.5	this work

vestigators (Table 3, and Cohn, M., unpublished). (b) Binding of Mg^{2+} to the EF-Tu · GDP complex causes a downfield shift of 0.5 ppm for the α-P and 1.3 ppm for the β-P. This compares with 0.8 ppm and 0.7 ppm for the α-P and β-P resonances, respectively, of GDP and GDP · Mg^{2+}, i.e. the influence of EF-Tu diminishes the influence of the metal ion on the chemical shift of the α-P by about 30%, whereas it increases the influence on the β-P by about 90%. (c) Binding of GDP[βS] to EF-Tu causes a shift of the α-P of 0.6 ppm downfield and 2.0 ppm downfield for the β-P resonance. (d) Binding of Mg^{2+} to the EF-Tu · GDP[βS] complex causes an upfield shift of 0.2 ppm (a shift of this magnitude is at the limit of experimental error) for α-P and a downfield shift of 2 ppm for the β-P. This compares with 0.5 ppm downfield for the α-P and 2.1 ppm downfield for the β-P of free GDP[βS] and GDP[βS]Mg^{2+}.

DISCUSSION

The results of our study show that the high stereoselectivity of EF-Tu for the configuration at the α-phosphorus atom of GDP[αS] is independent of the nature of the divalent metal ion and, moreover, is even expressed in the absence of metal ions. This is a strong indication that the protein interacts directly with one of the oxygens of the α-phosphate, and that this interaction is adversely affected by replacement of the oxygen by sulfur. According to this interpretation, the metal ion is not complexed to this phosphorous atom. Caution must be exercised in interpreting the results obtained with the thiophosphate analogs in the presence of Cd^{2+}, since the results in Fig. 3 show that with 1 mM Cd^{2+}, 75% of the GDP binding activity of EF-Tu is lost. Very low reaction rates in the presence of Cd^{2+} have also been observed for other nucleotide-utilizing enzymes (e.g. [2, 15]). Thus consideration must be queen both to the cause of the low reaction rates or low binding activities in the presence of Cd^{2+} and to which conclusions can be drawn about the nucleotide structure at the active site when thiophosphates are used in the presence of Cd^{2+}.

Our inhibition experiments also show that GDP[βS] is a fourfold better analog of GDP in the absence of metal ions than in its presence. This could be explained if GDP or GDP[βS] are bound to EF-Tu via two oxygens of the β-phosphate, thus leaving the sulfur free in the absence of metal ions. Addition of Mg^{2+} to the EF-Tu · GDP[βS) complex forces the sulfur into an unfavorable coordination either with the protein or with Mg^{2+} itself since Mg^{2+} is supposed to have a higher affinity for oxygen than for sulfur [2]. In the absence of metal ions, GDP[βS] still binds sixfold more weakly than GDP. However, on the basis of our tentative model, a factor of three would be expected on statistical grounds alone since, of three possible rotational situations of the β-phosphate group in GDP[βS] which would bring either oxygens or sulphur to the sites of interaction with the protein, only one will allow interaction with the two oxygens.

This leads us to propose the schematic model of the binding of GDP to the active site of EF-Tu shown in Fig. 9. In this the protein binds directly to one oxygen of the α-phosphate with the stereochemistry at the phosphorous atom as shown, and to two of the β-phosphate oxygens; the metal ions is coordinated only to the β-phosphate and the enzyme. On the basis of this scheme, one could predict that, in the presence of Cd^{2+}

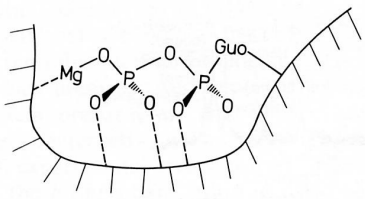

Fig. 9. *Model of the binding of GDP to the active site of EF-Tu as discussed in the text.* The continous black line represents the active site of the protein. The choice of stereochemical arrangement of groups around the α-phosphate is based on the specificity of GDP[αS] binding. The absolute stereochemistry of the diastereomers is known by analogy with the isomers of ADP[αS] [4, 23]

with its high affinity towards sulfur, GDP[βS] should be a better analog than in the presence of Mg^{2+}, which is in fact so (Fig. 3). However, the relative affinities of the thiophosphate analogs are all higher in the presence of Cd^{2+}, so that this result should be interpreted with caution.

Rubin et al. [16] have found, by X-ray structural analysis of EF-Tu from *Escherichia coli*, that the α and β-phosphates of GDP are hydrogen-bonded to side-chain residues of the protein, none of which can be identified at the present moment. These authors have told us however, that they find the β-phosphate has much more intimate contact with the protein than the α-phosphate, which would agree with our model. The fact that an unusually large chemical shift for the β-phosphate of GDP on binding to EF-Tu has been observed by ^{31}P-NMR [18–20] (see also below) is in our view a further indication of the tight interaction between the protein and the GDP β-phosphate.

It is of interest to note that certain similar conclusions have been drawn concerning the structure of the myosin-ADP-Mg^{2+} complex [21]. In this case, the nucleotide also appears to be bound as the β-monodentate complex, and preference for the A diastereoisomer of ADP[αS] results from an interaction of the α-phosphate with the enzyme. The poor interaction of both ADP[βS] and ATP[βS] with the enzyme is interpreted to indicate an interaction of the β-phosphate group with the enzyme, but data on the binding of ADP and the analogs in the absence of metal ions have not been obtained, so that a direct comparison with EF-Tu concerning the binding of the β-phosphate group is not possible.

The results obtained by inhibition experiments find strong support in the magnetic resonance measurements. Firstly, the broadening of the EPR spectra of the Mn^{2+} after formation of the EF-Tu · nucleotide · Mn^{2+} complex as compared to the EF-Tu · Mn^{2+} complex suggests that Mn^{2+} is indeed coordinated in some way to the nucleotide. Also, the affinity of Mn^{2+} for EF-Tu · GDP is about one order of magnitude higher than its affinity for EF-Tu [7]. These two pieces of evidence taken together suggest that Mn^{2+} is associated with the nucleotide in the EF-Tu · GDP · Mn^{2+} complex. The similarity of the spectra when either GDP or GTP is bound suggests that the coordination pattern may be similar in the two complexes, as has also been observed for analogous complexes of EF-Tu from *E. coli* [22]. Moreover, it may be concluded from the unchanged spectral type of the manganese spectrum that the symmetry of the coordination sphere of Mn^{2+} is not greatly disturbed by the interaction with the nucleotides. Secondly, the EPR experiments using the ^{17}O analogs of GDP indicate that Mn^{2+} is coordinated to the β-phosphorous, but not to the α-phosphorous group. The line broadening of the Mn^{2+} resonance in the EF-Tu · Mn^{2+} complex with [β-$^{17}O_4$]GDP, which does not show up in the EF-Tu · Mn^{2+} · [α-$^{17}O_3$]GDP complex, is very probably due to the superhyperfine interaction between the unpaired electron of Mn^{2+} and the ^{17}O nucleus [12, 13].

The interpretation of the ^{31}P-NMR results leads to the same conclusions. The influence of EF-Tu on the chemical shifts of the ^{31}P resonances of GDP and GDP[βS] is larger for the β-phosphorous in both cases. In addition, for both nucleotides, the influence of the metal ion on the chemical shift of the α-phosphorus is diminished by EF-Tu and greatly enhanced for the β-phosphorous. Also, Mg^{2+} binds much more strongly to the EF-Tu · GDP complex than to the EF-Tu · GDP[βS] complex. This is in agreement with the suggestion made above that there are important interactions between two of the oxygens of the β-phosphorous group and the protein. This would force Mg^{2+} to bind to the sulfur of the thiophosphate group in the EF-Tu · GDP[βS] complex, and it is known that this interaction is weak. Although it is not easy to interpret chemical shifts, especially of ^{31}P, in terms of interaction of atoms or group of atoms with metal ions, the results of the NMR experiments may nevertheless be taken to indicate that Mg^{2+} in the EF-Tu · GDP · Mg^{2+} complex interacts more strongly with the β than with the α-phosphorous group.

We have pointed out the apparent similarity between MgADP binding to myosin and MgGDP binding to EF-Tu. This idea has been strengthened by the recent confirmation of the β-monodentate MgADP structure at the active site of myosin using both the thiophosphate [21] and EPR methods [24]. It is also of interest to note that the two independent methods for determining the involvement of the α and β-phosphate groups with the metal ion at the active site have been in agreement in the three cases in which they have been compared, namely for EF-Tu [14] (and this work) and myosin [21, 24], both of which bind the β-monodentate complexes of GDP and ADP respectively, and creatine kinase [12, 15] which shows an interaction of the metal ion with both the α and β-phosphates. This agreement between the two techniques enhances the degree of confidence which can be placed in either of them.

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