Georgia Journal of Science

Volume 63 *No. 2 Scholarly Contributions from the Membership and Others* | Article 10

2005

31P NMR Study of GTP-Cation Interactions

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Running Title: ^{31}P NMR of GTP

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ABSTRACT

Guanosine 5'-triphosphate (GTP) plays a pivotal role in many biological processes. The interactions of cations with GTP are essential to the stabilization of this nucleotide and the regulation of its functions. In this study, we present solution ^{31}P NMR data on GTP samples in varied salt concentrations or pHs. From chemical shifts and T_1, T_2 relaxations, it is concluded that the interaction strengths of cations with GTP is in the order of $Ca^{2+} > Mg^{2+} > Li^{+} > Na^{+}$ for the cations, and in the order of $\beta > \gamma > \alpha$ for the phosphate groups; An increased sample pH causes deprotonation of the β and γ phosphates with enhanced cations' affinities, as indicated by significant chemical shift changes, while the chemical shift of α phosphate is essentially unaffected by varying pH. These results will aid in exploring the mechanism of GTP-cation interactions, and fully understanding the effects of cations on GTP's stabilization and its biological activities.

Key Words: ^{31}P NMR; chemical shifts; relaxations; GTP-cation interactions

INTRODUCTION

Guanosine 5'-triphosphate (GTP) is involved in many biological activities, such as the citric acid cycle and the activation of guanine nucleotide-binding proteins (G-proteins), which typically switch between active GTP- and inactive GDP- bound states to regulate a variety of cellular processes including cell growth, apoptosis and differentiation, etc. (1-4). Regulation of cations' binding to GTP is essential for the stabilization of GTP and GTP-mediated functions (5-7). For example, Na^{+} and Li^{+} ions have been shown to induce an inhibition of GTP-stimulated protein activities in vitro (8,9). Mg^{2+} is believed to play two distinct roles in Ras-GTP system, i.e. as a conformational regulator through its interactions with the substrate and as a key element for the GTP hydrolysis, while other divalent cations such as Ca^{2+} may have no such effect (10,11).

Nuclear magnetic resonance (NMR) has been widely used in the cation-bound GTP or GTP-bound protein systems to obtain various structural and dynamic information, such as the structural changes of proteins induced by bound GTP or GTP analogs, the mechanism of G-protein activation, the effects of cations on GTP's affinity to proteins, the process of GTP hydrolysis to GDP, and the competitive bindings of different cations to the nucleotides (12-21).

In this publication, we present ^{31}P NMR spectroscopy of GTP in varied salt concentrations and pHs. The ^{31}P chemical shift and T_1, T_2 relaxation data are correlated to characterize the interactions between GTP and different cations (Li^+, Na^+, Mg^{2+}, Ca^{2+}), and their affinities for the α, β, and γ sites of GTP (see Figure 1). Some structural and dynamic features of GTP in relation to cations' bindings are analyzed.

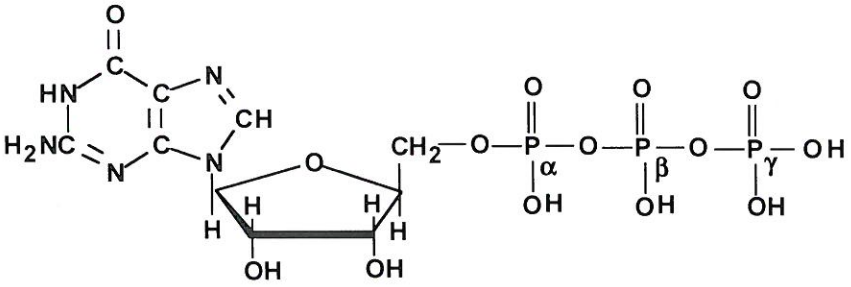

Figure 1. GTP molecule.

MATERIALS AND METHODS

GTP and all other chemicals were purchased from Fisher Scientific/Acros Organics (New Jersey, USA), which were of analytical purity and used without further purification. NMR samples were prepared by dissolving GTP in D_2O for the purpose of field lock. To study clear effects of cations on GTP, each of the salts LiCl, NaCl, $MgCl_2$ or $CaCl_2$ was dissolved in 10 mM GTP solutions with varied salt concentrations in the range of 10 - 500 mM. To study clear effects of pH variation on GTP, the sample pHs were adjusted in the range of pH 2.5 - 10.5 using 1 M NaOH and HCl solutions and were monitored by a pH meter (Denver Instrument).

^{31}P NMR experiments were conducted at room temperature, using a Varian mercury-200 spectrometer operating at spectral frequency 81.015 MHz, and a 4-nucleus auto-NMR probe (suited for ^1H, ^{19}F, ^{13}C, ^{31}P) with 5 mm sample tubes. The 1D spectra were acquired with typically 14.5 μs of $\pi/2$ pulse length, 960 scans and 3 s repetition delay. The spin-lattice relaxations T_1 of phosphorus nuclei were measured using the inversion-recovery method i.e. $\pi - \tau - \pi/2$ pulse sequence, with typically 96 scans and incremented delays τ in range of 0.1 ms -10 s. The spin-spin relaxations T_2 were measured using

the spin-echo method i.e. $\pi/2 - \tau - \pi - \tau$ pulse sequence, with typically 360 scans and incremented delays τ in range of 0.1 ms -10 ms. The chemical shift values of GTP (ppm) were referenced to 85% phosphoric acid. Peak integration was performed to obtain intensity of each resonance peak on T_1 and T_2 spectra, and the results were fit into the corresponding equations, $S_1(\tau) = S_0 [1 - 2 \exp(-\tau / T_1)]$ and $S_2(\tau) = S_0 \exp(-2\tau / T_2)$, from which T_1 and T_2 values were deduced.

RESULTS

Figure 2 shows representative ^{31}P spectra of Na-GTP (A), Li-GTP (B), Mg-GTP (C) and Ca-GTP (D), obtained with GTP samples at a lower salt concentration (10 mM NaCl, LiCl, $MgCl_2$ or $CaCl_2$) and pH 2.5. The spectral assignment of α, β, γ phosphates are denoted. These spectra are well resolved, characterized by doublets for both α and γ resonances, and a triplet for β resonance. The effects of cation species on chemical shifts of GTP are clearly seen from the spectra. Relative to Na-GTP (spectrum A), the interactions of Li^+, Mg^{2+} and Ca^{2+} with GTP induce more or less down-field shifts of the β and γ peaks. This is particularly true for Mg-GTP and Ca-GTP in spectra C and D. However, the resonance frequency of α phosphate remains essentially the same in all these spectra.

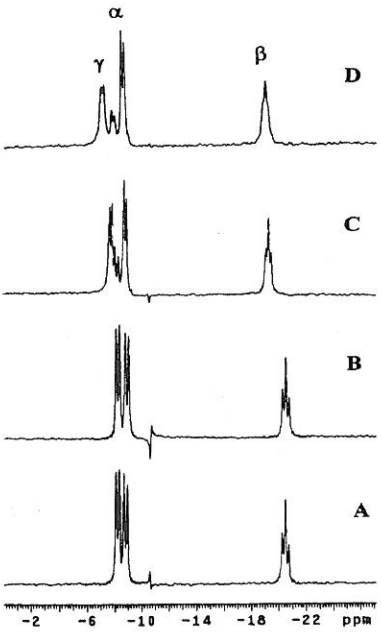

Figure 2. ^{31}P spectra of Na-GTP (**A**), Li-GTP (**B**), Mg-GTP (**C**) and Ca-GTP (**D**) at lower salt concentration, acquired with 10 mM GTP in 10 mM Na^+, Li^+, Mg^{2+} or Ca^{2+} solutions at pH 2.5.

Figure 3 shows spectra of Na-GTP (A), Li-GTP (B), Mg-GTP (C) and Ca-GTP (D), obtained with GTP samples at a higher salt concentration (300 mM NaCl, LiCl, $MgCl_2$ or $CaCl_2$) and pH 2.5. Due to increased cation-GTP interactions at high salt concentration, the spectra are characterized by significant peak broadening as compared to Figure 2, in particular the β and γ peaks of Ca-GTP, and to a less extent, the β and γ peaks of Mg-GTP and Li-GTP, respectively.

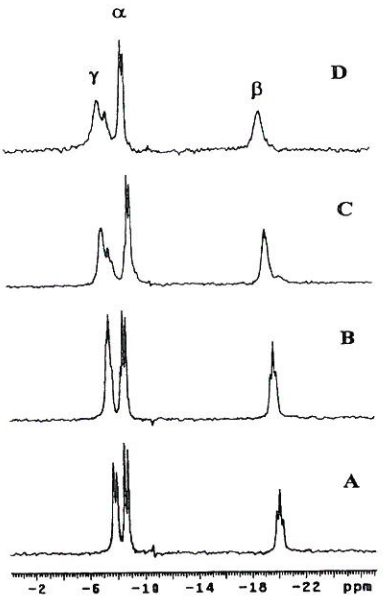

Figure 3. ^{31}P spectra of Na-GTP (**A**), Li-GTP (**B**), Mg-GTP (**C**) and Ca-GTP (**D**) at higher salt concentration, acquired with 10 mM GTP in 300 mM Na^+, Li^+, Mg^{2+} or Ca^{2+} solutions at pH 2.5.

The effects of cations on chemical shifts of phosphates, obtained from ^{31}P spectra of GTP at different salt concentrations (10 - 500 mM of Na^+, Li^+, Mg^{2+} or Ca^{2+}) and pH 2.5, are further illustrated in Figure 4. The enhanced cations' interactions with β and γ phosphates of GTP at increased salt concentrations cause a nuclear deshielding, leading to noticeable down-field shifts of the β and γ resonance frequencies (Figures 4B and 4C, respectively). In contrast, the increase in salt concentration induces slightly down-field shifts for the α resonance of Na-GTP and Li-GTP but up-field shifts for the α resonance of Ca-GTP and Mg-GTP (Figure 4A).

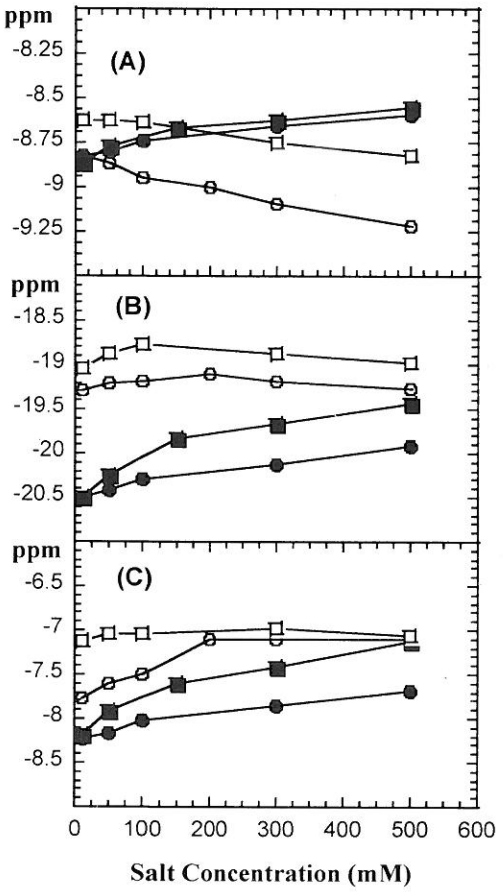

Figure 4. ^{31}P chemical shifts of Na-GTP (●), Li-GTP (■), Mg-GTP (○) and Ca-GTP (□) in varied salt concentrations. (**A**): α phosphate; (**B**): β phosphate; and (**C**): γ phosphate.

The different affinities of cations can also be manifested by the spin-lattice relaxations of GTP phosphates. Figure 5 represents the relaxation times T_1 (in seconds) of GTP at varied salt concentrations. The increase in salt concentrations lowers T_1 relaxations of α (Figure 5A), β (Figure 5B) and γ nuclei (Figure 5C) respectively, but such effects to reduce T_1 are different, with an order of $Ca^{2+} > Mg^{2+} > Li^{+} > Na^{+}$. By comparing these figures, it is found that the T_1 relaxations of α, β and γ phosphates are in an order of $\beta < \alpha < \gamma$ at 0 mM salts; with increased cations' interactions at high salt concentrations, however, the spin-lattice relaxation times of α, β, γ phosphates are shortened differently, so that the relative T_1 values become $\beta < \gamma < \alpha$.

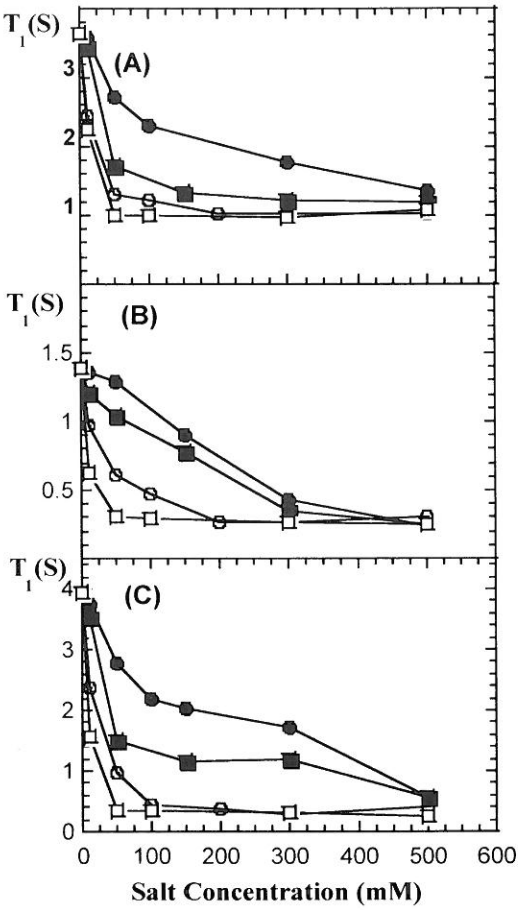

Figure 5. T_1 relaxations (in second) of Na-GTP (●), Li-GTP (■), Mg-GTP (○) and Ca-GTP (□) in varied salt concentrations. (**A**): α phosphate; (**B**): β phosphate; and (**C**): γ phosphate.

Our NMR studies also reveal the chemical shift variations at different sample pHs. Figure 6 shows the data for Li-GTP and Mg-GTP. The change of pH from ~2.5 to ~8.0 significantly increases the chemical shift values of β and γ phosphates of Mg-GTP and Li-GTP, and further increase of pH above ~8.0 has only a small effect. But the varying pH has little effect on the chemical shift of α phosphate. In addition, the variations in chemical shifts at higher pH may accompany changes in relaxations of GTP. Figures 7 and 8 show the representative T_1 and T_2 spectra. The relaxation times for α, β and γ phosphates of Li-GTP and Mg-GTP, obtained from GTP samples in 50 mM Li^+ or Mg^{+2} solutions at pH 7, are illustrated in Figure 9.

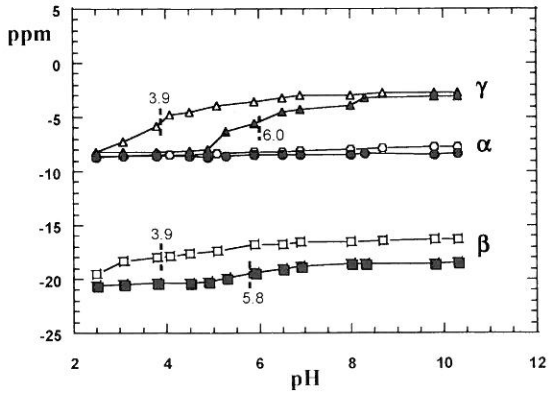

Figure 6. ^{31}P chemical shifts of Li-GTP: α (●), β (■), γ (▲) phosphates, and Mg-GTP: α (○), β (□), γ (Δ) phosphates. The results were obtained with 10 mM GTP in 10 mM Li^+ or Mg^{2+} solutions at varied pHs.

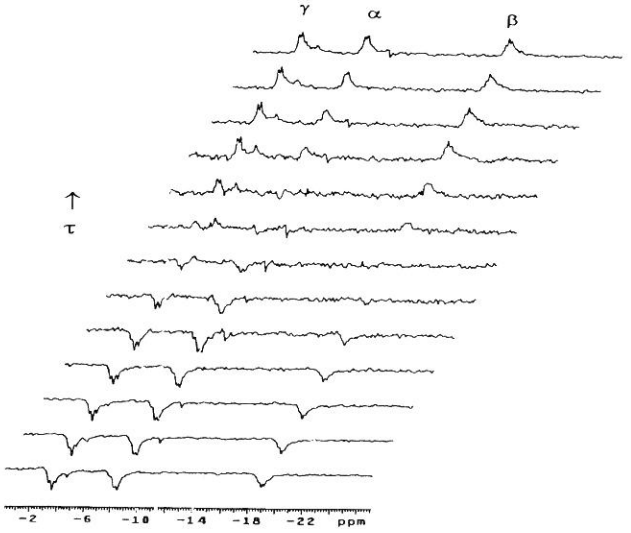

Figure 7. Representative T_1 spectra of GTP acquired with π - τ - π/2 pulse sequence, where τ values were 0.0001, 0.001, 0.01, 0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.4, 0.5, 0.7, and 0.9 s.

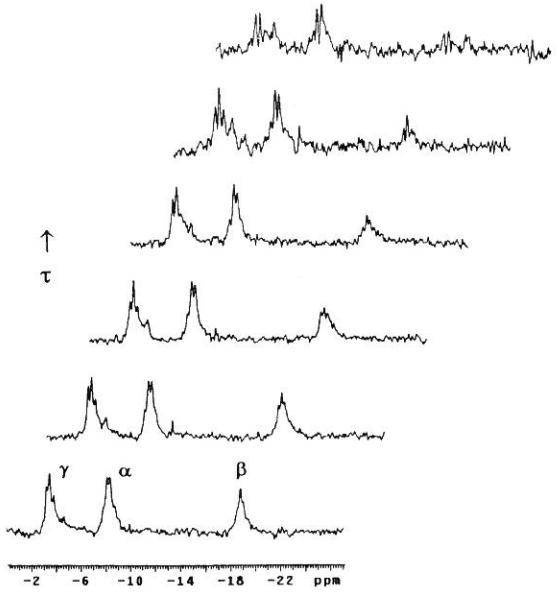

Figure 8. Representative T_2 spectra of GTP acquired with $\pi/2 - \tau - \pi - \tau$ pulse sequence, where τ values were 0.1, 1, 2, 4, 6, and 8 ms.

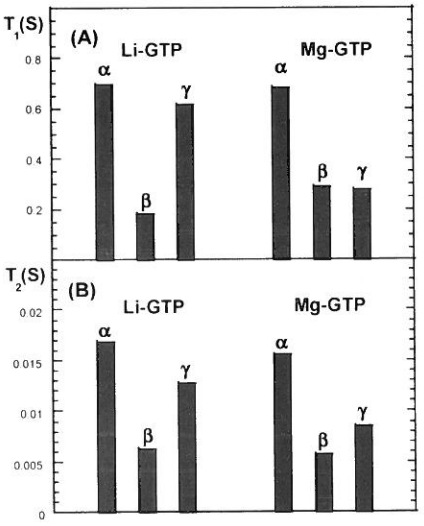

Figure 9. T_1 (**A**) and T_2 (**B**) relaxations (in seconds) of α, β and γ phosphates of Li- and Mg-GTP. The results were obtained with 10 mM GTP in 50 mM Li^+ or Mg^{2+} solutions at pH 7.

DISCUSSION

The NMR resonance frequency ω depends on the Larmor frequency ω_0 and nuclear shielding σ at the nuclear site, $\omega = \omega_0 (1 - \sigma)$. Based on the spectra in Figure 2, it can be concluded that the nuclear shielding σ at the three phosphate sites is in the order of $\beta > \alpha > \gamma$, with β phosphorus nucleus being the most shielded and γ nucleus the least shielded. As seen from the GTP structure (Figure 1), the β phosphate is adjacent to two phosphate groups (α, γ), α phosphate is adjacent to one phosphate group (β) and a sugar ring, while γ phosphate is only connected to one phosphate group (β). Apparently, both the adjacent phosphate group(s) and sugar ring contribute to the nuclear shielding of phosphorus, while the former has greater shielding effect than the latter. From the resonance splitting in Figure 2, i.e. the doublet for α or γ peak and the triplet for β peak, the through-bond spin coupling constant, corresponding to a two-bond separation of P–O–P in this case, can be evaluated as $^2J \approx 19$ Hz.

The down-field shifting of β and γ peaks of Mg-GTP (Figure 2C) and Ca-GTP (Figure 2D), as compared to Na-GTP (Figure 2A) and Li-GTP (Figure 2B), gives a clear indication that Mg^{2+} and Ca^{2+} ions have a stronger deshielding effect on the β and γ nuclei of GTP than that of Na^+ and Li^+. This may imply stronger cation affinities of Mg^{2+} and Ca^{2+} for the β and γ phosphates. However, the resonance frequency of α peak is essentially unchanged across the spectra 2A-2D, suggesting that the interactions of these cations with α phosphate are all weak at the sample condition of low salt concentration and pH.

In addition, different peak widths noticeable on our GTP spectra provide another hint for analyzing the relative strengths of GTP-cation interactions. In Figure 2, the spectrum D has broader β and γ peaks, suggesting a stronger Ca^{2+} interaction than Na^+, Li^+ and Mg^{2+}. Such spectral broadening becomes more pronounced in Figure 3, due to increased interactions of these cations with GTP phosphates at higher salt concentration. As observed from spectra 3A-3D, the effects of cations' interactions on peak broadening have in general an order of Ca-GTP > Mg-GTP > Li-GTP > Na-GTP, and an order of $\beta > \gamma > \alpha$ for the GTP phosphate groups.

Figure 4 further reveals the effects of GTP-cation interactions on the chemical shifts of GTP phosphates. With the increase of salt concentrations, a down-field shift of β or γ phosphates of Mg-GTP and Ca-GTP is more dramatic than that of Na-GTP and Li-GTP, as characterized by a steeper rise of the curves representing β and γ phosphates of Mg-GTP and Ca-GTP in Figures 4B and 4C. In the salt concentration range of 50 - 100 mM, the effect of Ca^{2+} on β and γ phosphates appears "saturated" and further increase of Ca^{2+} concentration causes no significant shift on either β or γ resonance of Ca-GTP. Over the entire range of salt concentration in Figure 4, the β shifts of Na-, Li-, Mg- and Ca-GTP are increased by ~ 0.7, 1.2, 1.4 and 1.7 ppm, respectively, as compared to their values at 0 mM salts (Figure 4B), whereas the γ shifts are increased by ~ 0.6 ppm for Na-GTP and ~1.2 ppm

for Li-, Mg- and Ca-GTP, as compared to that at 0 mM salts (Figure 4C). In contrast, the effects of these salts on chemical shift of α phosphate are much smaller, with a maximum change of about 0.2 - 0.4 ppm over the entire salt concentration range (Figure 4A). Moreover, the increase of salt concentrations has strikingly different effects on the α shift of Na-, Li-, Mg- and Ca-GTP. As the increase of salt concentrations, the resonance frequency of α phosphate experiences slightly down-field shifts for Na- and Li-GTP, but slightly up-field shifts for Ca-GTP and Mg-GTP, respectively.

According to Tachuchi et al., the binding of divalent cations to ATP phosphates may have two possible forms, i.e. β-, γ-bidentate and α-, β-, γ-tridentate complexes (22). This may be true for GTP as well. According to Kolondy et al., an up-field shift of phosphate resonance can be caused by a change in O-P-O bond angle (23). We believe that the α phosphate of Ca-GTP and Mg-GTP may experience such O-P-O angle change as a α-, β-, γ-tridentate form of GTP-cation interaction is involved, resulting in a nuclear shielding on α site, which also exceeds the possible deshielding effect because of the lower affinity of cations to the α phosphate. Therefore an overall up-field shift is observed. The relatively higher extent of up-field shift for α resonance of Mg-GTP, as compared to Ca-GTP, might be attributed to a smaller ionic radius of Mg^{2+} than Ca^{2+}, thus a greater change in O-P-O angle of α phosphate in the tridentate interaction.

By inspecting the relaxation data in Figure 5, it is found that the effects of cation interactions on reducing T_1 are generally in an order of $Ca^{2+} > Mg^{2+} > Li^+ > Na^+$. This also agrees with the results from 1D spectra, as discussed above. As for different phosphate sites of α (Figure 5A), β (Figure 5B), and γ (Figure 5C), at 0 mM salts the T_1 values are in an order of $\beta < \alpha < \gamma$, i.e. the spin-lattice relaxation rates are $\beta > \alpha > \gamma$, the same order as for the nuclear shielding on these sites. Due to increased cation interactions at high salt concentrations, however, the T_1 values become $\beta < \gamma < \alpha$, i.e. the spin-lattice relaxation rates become $\beta > \gamma > \alpha$, an order which is in agreement with the chemical shift analysis. Thus the correlation of these chemical shift and relaxation data strongly suggests that the GTP-cation interactions are in an order of $Ca^{2+} > Mg > Li^+ > Na^+$ for cations, and in an order of $\beta > \gamma > \alpha$ for the GTP phosphates.

The interactions of cations with GTP phosphates are dominated by electrostatic attraction. The charges and radii of cation species may contribute significantly to the strengths of their interactions. Earlier, Korolev et al. studied the competitive binding of cations to oriented DNA fibers by ion exchange and Monte Carlo simulation, and found that the binding affinities of cations to oriented DNA followed an order of $Ca^{2+} > Mg^{2+} > Na^+ > Li^+$, where a higher Na^+ affinity than Li^+ was explained by a specific (non-electrostatic) binding mode for Na-DNA (24,25). This order on cations' binding to DNA is generally in agreement with the present NMR studies on the strengths of GTP-cation interactions.

The chemical shift variations of GTP at different sample pHs (Figure 6) can be well explained by the combination effects of cations' affinity for phosphates and the protonation / deprotonation of GTP phosphate groups. At low pH range, the α, β and γ phosphates of GTP are protonated, and a cation's affinity to phosphate groups is somewhat limited by the protonation. With increased pH, the resonance frequencies of β and γ phosphates experience increased down-field shifting, which can be attributed to the deprotonation of β and γ phosphates, and thus more favorable to a cation's binding. In contrast, the resonance frequency of α phosphate is almost unchanged over the entire pH range. This implies that the α phosphate essentially remains in a protonated state even at a high pH range and cation's affinity for α phosphate is relatively low. These chemical shift variations of GTP at varying pHs are qualitatively in agreement with those reported for adenosine triphosphate and diadenosine tetraphosphate (23,26).

Using an approach similar to the Henderson-Hasselbalch equation, pH = pKa − log ([acid]/[base]), we estimated the transition point between the protonated state and deprotonated state of β or γ phosphate as the midpoint on the rise of β or γ curve, at which pKa = pH. The obtained pKa values are ~3.9 for β and γ phosphates of Mg-GTP and ~5.8 and 6.0 for β and γ phosphates of Li-GTP, as denoted in Figure 6. Notice that the γ phosphate group may involve two steps of protonation / deprotonation transition, however, the current NMR measurements only provide us with one observable transition step for the γ phosphate. Over the entire pH range, Mg-GTP shows greater chemical shift values and lower pKa than Li-GTP, in accordance with stronger Mg^{2+} affinity for phosphates than Li^{+}.

It is expected that enhanced cation interactions with deprotonated GTP must accompany faster relaxation rates. This is confirmed by the T_1 data of Li-GTP and Mg-GTP obtained at pH 7 (Figure 9), which are much shorter than those at pH 2.5 (Figure 5). Figure 9 also gives a comparison of T_1 (A) and T_2 (B) data obtained at the same sample conditions.

According to NMR theory, the nuclear spin relaxation rates of phosphorous are contributed by various relaxation mechanisms, including the dominant spin-spin dipolar interaction, and chemical shift anisotropy, which depends on the magnetic field strength (27, 28). In rapid molecular motion, or the so called "extreme narrowing region," $T_1 / T_2 \approx 1$. However, our preliminary results in Figure 9 demonstrate that the ratios T_1 / T_2 of Li-GTP and Mg-GTP are all much greater than 1 for α, β and γ sites. This suggests that the overall rotational motion of GTP molecule or phosphate local motion appears to be slow and phosphate linkages are not flexible at the experimental conditions. Strong cation interactions may contribute to the slow motion of GTP. We will continue our investigation on this subject.

In conclusion, these ^{31}P chemical shifts and relaxation data provide valuable information on cations' interactions with GTP phosphates. The relative strengths of their interactions can be deduced as $Ca^{2+} > Mg^{2+} > Li^{+} > Na^{+}$ for the cations and β > γ > α for GTP phosphates. At increased sample pHs, β

and γ phosphates are deprotonated, but the deprotonation is much less for the α phosphate. Stronger cation bindings are expected for deprotonated state of GTP. The molecular motion of GTP appears slow at the experimental conditions. These results are important for exploring the mechanism of GTP-cation interactions, assessing the effects of cations on GTP's stabilization, and further understanding GTP's biologically relevant activities.

ACKNOWLEDGEMENTS

This work was supported by a NIH/NIGMS grant (No. 5 S06 GM 060314-03) awarded to Z. Song.

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