^{31}P NMR SPECTRA OF THIOPHOSPHATE ANALOGUES OF GUANOSINE NUCLEOTIDES

pH dependence of chemical shifts and coupling constants

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1. Introduction

Various enzymes use the guanosine nucleotides GMP, GDP and GTP as natural substrates rather than the more common adenosine nucleotides AMP, ADP and ATP. Hydrolysis of a GTP molecule accompanies, for example, the elongation step of protein synthesis on ribosomes. Some ^{31}P NMR studies on adenosine nucleotides bound to enzymes have been done [1]. The study of the phosphorothioate of AMP as a substitute for the unmodified nucleotide has proved to be useful in exploring the mechanism of glycogen phosphorylase because the difference of the ^{31}P chemical shifts of the nucleotide and the corresponding phosphorothioate facilitated the distinction of the two in the ^{31}P NMR spectrum [2]. This difference in chemical shifts makes the phosphorothioates extremely attractive for structural as well as kinetic studies of enzyme-bound nucleotides by NMR methods, especially in light of the seemingly very similar course of reaction of nucleotides and their phosphorothioates as exemplified for glycerol kinase [3].

Besides their biochemical applications, these derivatives merit some interest: analogs of the adenosine nucleotides exhibit a larger change in the chemical shifts of all phosphorus atoms in the nucleotide on secondary protonation of the terminal phosphate, and the values of the coupling constants between the phosphorus atom to which the sulfur is bound and the adjacent phosphorus atoms are increased by a considerable amount in the adenosine nucleotides [4].

To provide a set of data useful for further ^{31}P NMR studies of the interaction of guanosine nucleotides with proteins as well as to compare the features of the phosphate chain of guanosine and adenosine nucleotides and their phosphorothioates, we examined the ^{31}P NMR spectra of these substrates at various pH values.

2. Materials and methods

Phosphorothioates were prepared according to [5]. We exclusively used the A form of the derivatives, as defined in [5], for our experiments. GMP, GDP and GTP were obtained from Pharma Waldhof (Düsseldorf). All nucleotides were passed over a Chelex 100 column to remove metal impurities and freeze-dried prior to use. All samples were in 50 mM Hepes, 0.1 mM EDTA buffer and contained 15–20% D_2O to provide a lock signal for the NMR instrument. PH was adjusted with DCl and KOH. The level nucleotides was always <12 mM to avoid mutual interaction.

NMR spectra were obtained on a Bruker HX 360 spectrometer working in the Fourier mode with a ^{31}P resonance frequency of 145.7 MHz. The temperature of the sample was regulated with a continuous flow of dry nitrogen and regulated to 15 + 1°C with a Bruker SV 200 temperature control unit.

Chemical shifts are expressed with respect to 85% H_3PO_4 as external reference at pH 7.0 and given with increasing numbers in the direction of decreasing field. All spectra were obtained with complete broad proton decoupling.

pK_a-values were determined with a least squares fit to:

$$\delta = \delta_{min} + \Delta \frac{10^{pH-pK}}{1 + 10^{pH-pK}}$$

with δ_{min}, Δ and pK as free parameters.

3. Results and discussion

Fig.1 shows the ^{31}P NMR spectra of GTP, GTPαS, GTPβS and GTPγS. As is the case for the analogs of the adenosine nucleotides, the ^{31}P resonances of the phosphorus atoms to which the sulfur is attached are shifted downfield by more than 50 ppm at low pH values. The changes in chemical shifts upon secondary protonation of the terminal phosphate follow the same pattern that was observed for the adenosine phosphorothioates [4]: a downfield shift for the resonances with the sulfur substituted for a nonbridge oxygen, an upfield shift for the others (fig.2, table 1). Complete data are not available for the changes in chemical shifts of the adenosine phosphorothioates, therefore a direct comparison, possibly revealing an influence of the adenosine and guanosine moieties on the phosphate chain, is not possible.

For the change of pK_a upon sulfur substitution the general rule holds that the closer the sulfur is to the terminal phosphate of GTP the lower is the secondary pK_a, i.e., the pK_a value follows the series: GTP > GTPαS > GTPβS > GTPγS. The same is valid for GDP, GDPαS, and GDPβS as well as GMP and GMPS.

The numerical values for the secondary pK_a values agree well with the corresponding ones of the adenosine nucleotides in the case of the triphosphates. They tend to be somewhat lower for the mono- and diphosphates of guanosine nucleotides, but this may well be due to the somewhat different experimental conditions rather than to the difference between the adenosine and guanosine moieties.

Table 2 shows the coupling constants, J, for the ^{31}P–O–^{31}P coupling between adjacent phosphorus atoms. Upon substitution of sulfur for a non-bridge oxygen, the coupling between the phosphorus atom concerned and the directly neighbouring phosphorus atom is increased by >7 Hz. The coupling constant between the β- and γ-phosphorus in GTPαS and α- and β-phosphorus in GTPγS is hardly changed by the substitution. An interesting pattern is found for the

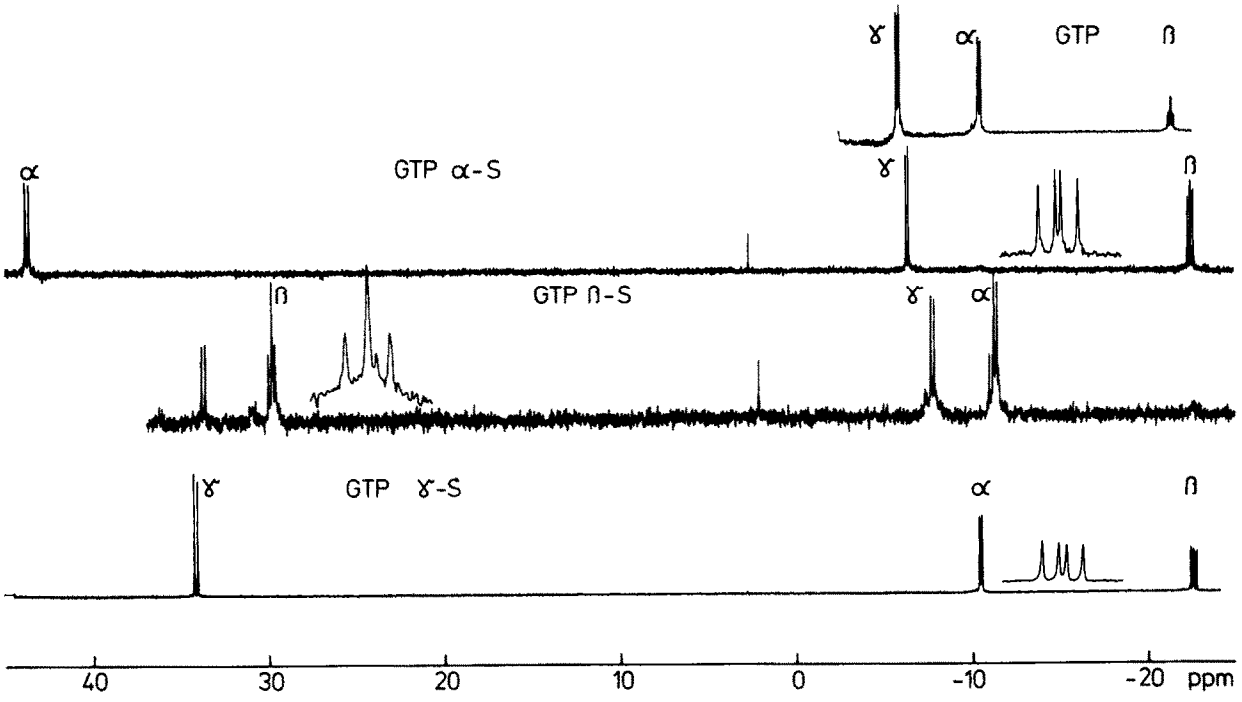

Fig.1. ^{31}P NMR spectra of GTP and its three phosphorothioates: 100 accumulations/spectrum; 60° pulse angle; repetition rate 5 s^{-1}; digital resolution 0.5 Hz; 16 kilobytes computer memory for GTP; 32 kilobytes for the phosphorothioates. Inserts show the resonances of the β-phosphates.

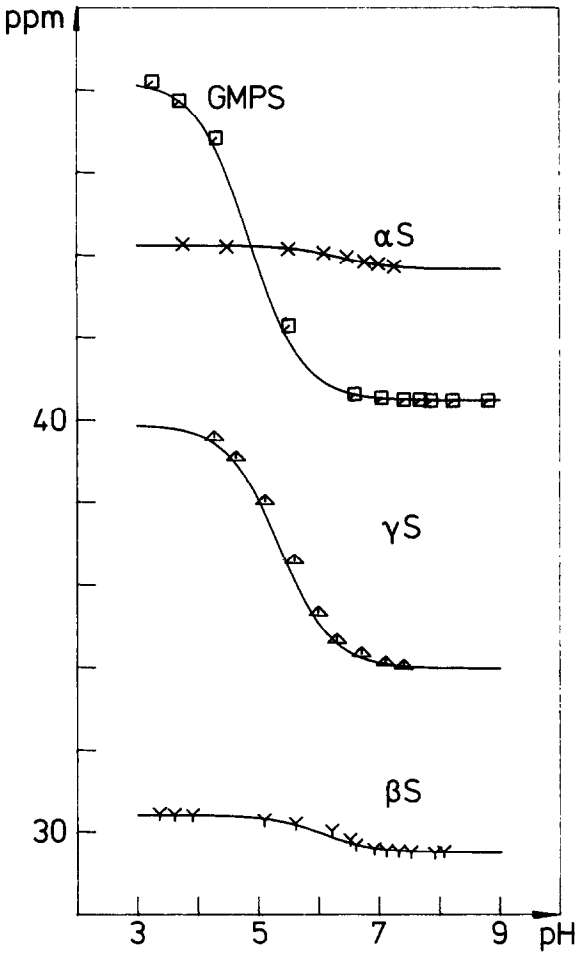

Fig.2. Titration curves of the sulfur labelled phosphates of GTPαS, GTPβS, GTPγS, and GMPS.

Table 1
pK_a values and chemical shifts of the ^{31}P resonances of GDP, GTP, and their phosphorothioates

	PK	δ_{AH}	δ_{A^-}	$\delta_{AH}-\delta_{A^-}$
GTP				
α		- 10.83	- 10.53	- 0.30
β	6.72	- 22.58	- 21.28	- 1.30
γ		- 10.24	- 5.57	- 4.67
GTPαS				
α		44.25	43.66	0.59
β	6.60	- 23.39	- 22.40	- 0.99
γ		- 10.20	- 5.66	- 4.54
GTPβS				
α		- 11.56	- 11.31	- 0.25
β	6.44	30.42	29.50	0.92
γ		- 11.06	- 5.95	- 5.11
GTPγS				
γ		- 10.75	- 10.54	- 0.21
β	5.47	- 23.44	- 22.69	- 0.75
γ		39.90	34.00	5.90
GDP				
α		- 10.73	- 10.18	- 0.55
β	6.44	- 10.20	- 5.81	- 4.39
GDPαS				
α		43.73	41.85	1.88
β	6.47	- 10.90	- 6.00	- 4.90
GDPβS				
α		- 11.64	- 11.31	- 0.33
β	4.97	39.55	33.53	6.2
GMP	6.26	0.85	4.40	- 3.55
GMPS	4.98	48.24	40.50	7.74

δ_{AH}, chemical shift in the protonated form; δ_{A^-}, chemical shift in the unprotonated form

The standard deviation for the chemical shift values is <0.05 in all cases

Table 2
Coupling constants of adjacent phosphorus atoms in guanosine nucleotides and their phosphorothioates

	J_{AH}	J_{A^-}	$J_{AH}-J_{A^-}$	J_{AH}	J_{A^-}	$J_{AH}-J_{A^-}$
GTP	19.4	19.4	0.0	19.6	19.6	0.0
GTPαS	27.0	27.0	0.0	19.5	20.8	- 1.3
GTPβS	26.7	26.7	0.0	27.1	27.1	0.0
GTPγS	20.0	20.0	0.0	28.1	29.7	- 1.6
GDP	20.3	22.3	-2.0			
GDPαS	26.9	30.1	-3.2			
GDPβS	28.0	31.8	-3.8			

J_{AH}, coupling constant in the protonated form; JA-, coupling constant in the deprotonated form

Standard deviations are <0.15 Hz in all cases except $J_{\alpha\beta}$ of GTPβS, where it is 0.6 Hertz

changes of the coupling constants under secondary protonation of the terminal phosphates (fig.3):

Neither $J_{\alpha\beta}$ nor $J_{\beta\gamma}$ change significantly during protonation of GTP. $J_{\beta\gamma}$ of GTPαS decreases by 1.3 Hz, $J_{\beta\gamma}$ of GTPγS decreases by 1.6 Hz, $J_{\alpha\beta}$ remains constant for these two phosphorothioates. In contrast, GTPβ exhibits no change for $J_{\beta\gamma}$ and $J_{\alpha\beta}$ within experimental error. The fact that $J_{\beta\gamma}$ and $J_{\alpha\beta}$ do not change in the latter case is probably due to the partially conserved symmetry of the phosphate chain in GTPβS.

GDP itself exhibits a change of its coupling con-

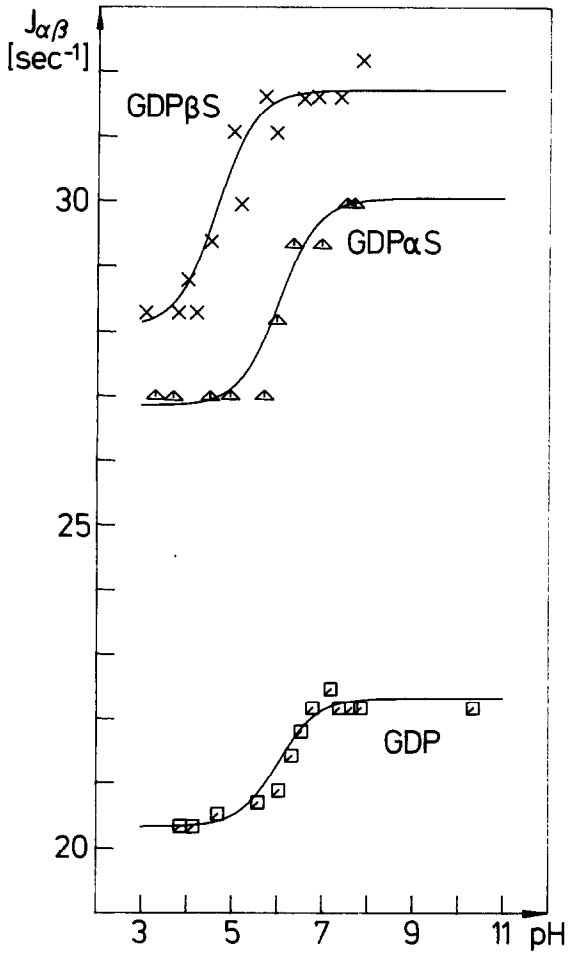

Fig.3. Titration curves of the coupling constant $J_{\alpha\beta}$ of GDP, GDPαS and GDPβS.

stant $J_{\alpha\beta}$ of −2 Hz under protonation of the β-phosphate. GDPαS shows a decrease of 3.2 Hz, i.e., 1.2 Hz more than GDP. GDPβS shows a decrease of 3.8 Hz, i.e., 1.8 Hz more than GDP. This resembles very closely the behaviour of GTPαS and GTPγs as compared to GTP.

For the sake of completeness it should be mentioned that a change in coupling constants $J_{\alpha\beta}$ and $J_{\beta\gamma}$ of ATP and $J_{\alpha\beta}$ of ADP has been in [6]. Because this is the only place in the literature known to the authors where such a change is mentioned this effect on ATP is probably due to the specific ionic conditions involved in those experiments.

Acknowledgements

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