^{31}P Nuclear Magnetic Resonance Spectra of the Thiophosphate Analogues of Adenine Nucleotides; Effects of pH and Mg^{2+} Binding†

Eileen K. Jaffe‡ and Mildred Cohn*,§

ABSTRACT: The ^{31}P nuclear magnetic resonance (NMR) spectra of the adenine nucleotide thio analogues, AMPS, ADPαS, ADPβS, ATPαS, ATPβS, and ATPγS, have been studied. Of primary interest were the increased sensitivity of chemical shifts to protonation and to magnesium binding of these analogues compared with the corresponding effects on AMP, ADP, and ATP. The usefulness of the characteristic NMR parameters of the thio analogues as probes in enzymatic reactions is discussed. The A^2 diastereoisomers of ADPαS and ATPαS and the A and B isomers of ATPβS were enzymatically synthesized and the diastereoisomers of ADPαS and ATPβS were distinguished by their ^{31}P NMR parameters. The stereospecificity of the enzymatic reactions involving the thio analogues of nucleotides can therefore be determined by ^{31}P NMR. The difficulty involved in assigning phosphate ligands of Mg in MgADP and MgATP and their analogues on the basis of the magnitude of chemical shift changes ($\Delta\delta$) induced by Mg binding upon each ^{31}P is discussed in the context of the anomalies in $\Delta\delta$ of each ^{31}P observed upon protonation of the terminal phosphate group. It is concluded that chemical shift data cannot yield unequivocal information concerning the absolute structure of metal complexes of nucleotides but can be used to monitor changes in metal chelation, for example, upon binding to enzyme.

NMR chemical shifts of ^{31}P have been used extensively to determine the pH in various cells and organs (Moon and Richards, 1973; Navon et al., 1977; Casey et al., 1977), the binding of Mg^{2+} to nucleotides in various systems (Cohn and Hughes, 1962; Casey et al., 1977) as well as structural features of enzyme–nucleotide complexes (Nageswara Rao and Cohn, 1977; Feldman and Hull, 1977). The secondary pK_as and ^{31}P chemical shifts of inorganic thiophosphate and thiophosphate analogues of adenine nucleotides differ greatly from the normal phosphates, although they are generally active substrates in enzymatic reactions with nucleotide substrates (Yount, 1975). Therefore a more extensive investigation of the characteristics of the ^{31}P NMR spectra of the adenine nucleotide thiophosphate compounds was undertaken to determine whether they would be advantageous probes in characterizing the pH dependence and structure of metal chelates in enzyme–substrate complexes and other more complex systems.

Some data on ^{31}P chemical shifts of AMPS[1] (Feldman and Hull, 1977) and of ATPαS, ATPβS, ATPγS, and ADPβS (Eckstein and Goody, 1976) and of the diastereoisomers of ADPαS and ATPαS (Sheu and Frey, 1977) have been reported. In the present work, the effects of pH and of magnesium binding on the chemical shifts and the spin–spin coupling constants of inorganic thiophosphate, AMPS, ADPαS, ADPβS, ATPαS, ATPβS, and ATPγS have been investigated. The enzymatic stereospecificity of the α- and β-S analogues of the nucleoside di- and triphosphates in a number of reactions (Eckstein and Goody, 1976) led us to attempt to distinguish the diastereoisomers of ADPαS and ATPβS[2] by their NMR parameters.

Materials and Methods

Adenosine 5′-*O*-thiomonophosphate (AMPS), adenosine 5′-*O*-(2-thiodiphosphate) (ADPβS), and adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS) were purchased from Boehringer Mannheim and used without further purification. The thiophosphate compounds contained less than 10% degradation products as determined by PEI cellulose thin-layer chromatography (Brinkman plates, 2 M LiCl) (Randerath and Randerath, 1964). The B form of ADPαS was a gift from Dr. F. Eckstein of the Max Planck Institute, Gottingen. All other thio analogues of ADP and ATP were synthesized enzymatically. ATP, ADP, AMP, potassium phosphoenolpyruvate, lithium potassium acetyl phosphate, Hepes buffer, and dithioerythritol were purchased from Sigma Chemical Co. Acetate kinase (*E. coli*) was purchased from Boehringer Mannheim; rabbit muscle pyruvate kinase and myosin were generously supplied by Dr. G. H. Reed and Dr. A. Weber, respectively; carp adenylate kinase was prepared by the method of Noda et al. (1975). The nitric acid used for pH titrations was Ultrapure grade (Ventron) to avoid paramagnetic impurities and $Mg(NO_3)_2$ was Merck Suprapure. Purified Na_3SPO_3 was a gift from Dr. G. D. Markham. Chelex 100 was purchased from Bio-Rad.

Preparation of Thiophosphate Analogues. ATPβS, diastereoisomer A, was prepared enzymatically as previously described (Eckstein and Goody, 1976). The course of the reaction was followed with ^{31}P NMR. The reaction mixture contained 20 mM ADPβS (10% AMP impurity), 25 mM phosphoenolpyruvate, 100 mM Hepes buffer pH 8.0, 25 mM $Mg(NO_3)_2$, 100 mM KCl, and 1 mM dithioerythritol in a final volume of 1 mL. With 1 mg/mL of pyruvate kinase, the reaction was complete in 25–30 min at 30 °C and was terminated by the addition of EDTA ~25 mM.

The B diastereoisomer of ATPβS was prepared by the ac-

† From the Department of Biochemistry and Biophysics, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104. *Received October 7, 1977.* This work was supported in part by Grant GM 12446 from the United States Public Health Service and Grant PCM 74-23620 from the National Science Foundation.

‡ Supported by a National Science Foundation Graduate Fellowship, 1975–1978.

§ Career Investigator, American Heart Association.

[1] Abbreviations used: AMPS, adenosine 5′-*O*-thiomonophosphate; ADPαS, adenosine 5′-*O*-(1-thiodiphosphate); ADPβS, adenosine 5′-*O*-(2-thiodiphosphate); ATPαS, adenosine 5′-*O*-(1-thiotriphosphate); ATPβS, adenosine 5′-*O*-(2-thiotriphosphate); ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; PEI, polyethylenimine.

[2] The nomenclature of the diastereoisomers of ATPαS, ATPβS, and ADPαS have been defined by Eckstein and Goody (1976).

0006-2960/78/0417-0652$01.00/0

etate kinase reaction as previously described (Eckstein and Goody, 1976). The reaction mixture contained 20 mM ADPβS (10% AMP impurity), 25 mM acetyl phosphate, 100 mM Hepes buffer, pH 7.4, 100 mM KCl, 25 mM $Mg(NO_3)_2$, and 0.3 mg/mL acetate kinase. The reaction was complete in 1 h at 30 °C. For measurements on the Mg complex, additional $Mg(NO_3)_2$ was added until no further spectral changes were apparent. EDTA (~ 60 mM) was added to remove magnesium, thus terminating the reaction and also making it possible to obtain the spectrum of the Mg-free nucleotide.

A solution of mixed diastereoisomers of ATPβS was synthesized from ADPβS by sequential reactions catalyzed by acetate kinase followed by pyruvate kinase. The reaction mixture contained 23 mM ADPβS in 100 mM Hepes, 100 mM KCl, 25 mM $MgNO_3$ in a volume of 1 mL. The solution was brought to pH 7.4 with KOH; 10 mM acetyl phosphate and 0.3 mg/mL acetate kinase were added and allowed to incubate for 30 min at 37 °C and form the B diastereoisomer. To form the A isomer from the residual ADPβS, the pH was adjusted to 8.0 and incubation was resumed with 15 mM phosphoenolpyruvate and 0.3 mg of pyruvate kinase for an additional 30 min. After a spectrum was obtained, 5 μmol of the A form of ATPβS from a previous preparation was added and those signals which intensified were assigned to diastereoisomer A.

ATPαS, the A form, was synthesized from AMPS by the coupled reactions of adenylate and pyruvate kinases. Since the stereospecificity of the pyruvate kinase reaction is for the A form of ADPαS (Eckstein and Goody, 1976), only the A form of ATPαS will be produced in the coupled reactions. In the adenylate kinase reaction, the stereospecificity had been established for ADPαS at the acceptor site, i.e., the ATP site, as the A form but the stereospecificity of the ADPαS at the donor site, i.e., the AMP site was not known. Since all of the ADPαS formed from AMP was converted to ATPαS, A form, then the ADP donor site of adenylate kinase must also be specific for the A form. While this work was in progress, similar experiments by Sheu and Frey (1977) were reported which agreed with this finding. The sequence of reactions is indicated below:

$$\text{AMPS} + \text{ATP} \overset{\text{adenylate kinase}}{\rightleftharpoons} \text{ADP}\alpha\text{S (A)} + \text{ADP} \quad (1)$$

$$\left.\begin{array}{l}\text{ADP}\alpha\text{S (A)}\\ \text{ADP}\\ \text{2 phosphoenolpyruvate}\end{array}\right\} \overset{\text{pyruvate kinase}}{\rightleftharpoons} \left\{\begin{array}{l}\text{ATP}\alpha\text{S (A)}\\ \text{ATP}\\ \text{2 pyruvate}\end{array}\right. \quad (2)$$

The initial concentration of ATP was very low (<3%) compared with AMPS and phosphoenolpyruvate. Consequently, ATP was cycled and high yields of ATPαS (A) were obtained. The net reaction was AMPS + 2 phosphoenolpyruvate → ATPαS + 2 pyruvate. The reaction mixture contained 20 mM AMPS, 0.5 mM ATP, 45 mM phosphoenolpyruvate, 25 mM $Mg(NO_3)_2$. Buffer, KCl, and dithioerythritol concentrations were the same as in the pyruvate kinase reaction described above for ATPβS (A) synthesis. The reaction mixture contained 0.2 mg of adenylate kinase and 0.1 mg of pyruvate kinase in 1 mL and the reaction was complete after 6 h of incubation at 37 °C. $Mg(NO_3)_2$ was added until no further spectral changes were apparent. Mg^{2+} was removed by successive washings with 0.5-mL portions of Chelex 100 before recording the spectrum of the metal-free nucleotide.

ADPαS, the A form, was prepared by the enzymatic degradation of ATPαS (A) using rabbit muscle myosin. The reaction mixture contained 10 mM ATPαS, 170 mM Hepes buffer, pH 7.5, 0.6 M KCl, 2 mM EDTA, and 0.15 mg of myosin in a volume of 1 mL. The reaction was complete after 3 h at 37 °C. The B form of ADPαS, received from Dr. F. Eckstein, was the residual material recovered after the pyruvate kinase reaction was run on chemically synthesized ADPαS. A solution of mixed diastereoisomers of ADPαS was prepared by adding 5 μmol of the B diastereoisomer to the solution of ADPαS (A) described above. $Mg(NO_3)_2$ was added until no further spectral changes were apparent.

NMR Measurements. ^{31}P spectra were recorded at 24.3 MHz on a Varian NV14 NMR spectrometer modified to operate in the Fourier transform mode and equipped with a multinuclear probe and with quadrature phase detection. A few spectra were recorded on a Bruker WH 360 operating at 145.7 MHz. The field was locked on deuterium and all spectra were recorded with broad band proton decoupling. At 24.3 MHz, temperature was regulated within ±2 °C with a Varian temperature controller NV 1480. Spectra were recorded on samples containing 0.4–1.0 mL (10–20% D_2O) and were measured in 8-mm tubes. All chemical shifts are expressed with respect to 85% H_3PO_4 as external reference.

The following conditions were used in obtaining all nucleotide thiophosphate spectra at 24.3 MHz: 2000-Hz sweep width; 2.0-s acquisition time; 60° pulse width; 1.6-Hz line broadening. Pulse delays varied from 1.0 to 4.0 s.

The ^{31}P spectra of the Mg complexes of the nucleotides AMPS, ADPβS, and ATPγS were recorded by adding sufficient $Mg(NO_3)_2$ to 20 mM solutions of the nucleotides containing 1 mM EDTA until no further spectral changes were apparent. For determination of pK_a values, all samples initially at alkaline pH were titrated stepwise with nitric acid. The samples contained 10–20% D_2O and the pH values reported are meter readings on a Radiometer pH meter 26.

Results

Effect of Sulfur Substitution on Chemical Shifts and Coupling Constants. The spectra of ATP, ATPαS (A), ATPβS (A), ATPγS, and MgATPγS in Figure 1 show the large downfield shift for that phosphorus of ATP on which a sulfur has replaced an oxygen. A chemical shift change of ~50 ppm downfield from the parent signal occurs when a nonbridge oxygen in a phosphate monoanion is replaced by a sulfur atom (cf. Figures 1A and 1B,C), and a downfield shift, ~40 ppm, occurs when a nonbridge oxygen in a phosphate dianion is replaced by a sulfur atom (cf. Figures 1A and 1D). The complete chemical shift, coupling constant, and pK_a data for all nucleotides and their magnesium complexes investigated are presented in Table I. From the data in Table I, the following generalizations concerning chemical shifts and coupling constants can be drawn. (1) The greater effect in the chemical shift (10 ppm) on monoanions than on dianions upon substitution of oxygen by sulfur shown in Figure 1 for ATP holds for ADP as well. Consequently, although the β-P resonances of ATP and ADP are separated by 16 ppm at alkaline pH, the β-P resonances of ATPβS and ADPβS are separated by only 4.0 ppm as illustrated in Figure 1C. (2) Phosphate–phosphate coupling constants (J_{PP}) in the thio analogues remain 20 ± 1 Hz as in ATP but phosphate–thiophosphate coupling constants (J_{PS}) are invariably much larger having values of 27.9 ± 1.4 Hz. Therefore, the β-P resonance of ATPβS appears as a triplet (Figures 1A and 1C) as with ATP where $J_{\alpha\beta} = J_{\beta\gamma}$, but the β-P resonances of ATPαS and ATPγS appear as a pair of doublets (Figures 1B and 1D) because J_{PS} is ~8 Hz > J_{PP}. The differences in chemical shift and in coupling constants between

TABLE I: Comparison of ^{31}P Chemical Shifts, Coupling Constants, and pK_as of Phosphate Compounds and Their Thio Analogues in the Presence and Absence of Magnesium (T = 18 ± 2 °C, Frequency 24.3 MHz).

Compounds	pH	NMR parameters: Chemical shift, ppm from 85% H_3PO_4 α-P	β-P	γ-P	Coupling constants (Hz) $J_{\alpha\beta}$	$J_{\beta\gamma}$	pK_a	−ΔpK_a Analogue − normal
P_i	8.0	−2.4 (s)[a]					6.7	
SP_i	8.0	−35.9 (s)					5.4	1.3
AMP	9.1	−4.1 (s)					6.5	
AMPS	9.1	−43.5 (s)					5.3	1.2
MgAMPS		−43.3 (s)						
ADP	8.0	9.7 (d)	5.3 (d)		21.7[c]		6.8	
MgADP	5.5	10.2 (d)	7.0 (d)		17.9			
ADPαS (A)[b]	7.3	−42.1 (d)	6.3 (d)		30.8			
MgADPαS (A)	7.6	−44.7 (d)	6.4 (d)		28.0			
ADPαS (B)[b]	7.3	−41.7 (d)	6.3 (d)		29.7			
MgADPαS (B)	7.6	−44.4 (d)	6.4 (d)		27.0			
ADPβS	8.0	11.1 (d)	−33.9 (d)		31.2		5.2	1.6
MgADPβS	8.0	11.0 (d)	−35.2 (d)		28.1			
ATP	8.0	10.9 (d)	21.3 (t)	6.0 (d)	19–20	19–20	6.7	
MgATP	8.0	10.7 (d)	19.1 (t)	5.5 (d)	15–16	15–16	5.3	
ATPαS (A)[b]	8.0	−43.4 (d)	22.2 (sd)	5.5 (d)	27.5	20.5		
MgATPαS (A)	8.0	−45.4 (d)	20.6 (sd)	5.8 (d)	26.1	15.8		
ATPβS (A)[b]	8.1	11.4 (d)	−29.9 (t)	6.0 (d)	26.5	27.3	6.5	0.2
MgATPβS (A)	8.0	11.2 (d)	−36.3 (t)	5.7 (d)	28.1	27.5		
ATPβS (B)[b]	8.1	11.4 (d)	−30.0 (t)	6.0 (d)	26.5	27.3		
MgATPβS (B)	8.0	11.2 (d)	−35.9 (t)	5.8 (d)	28.1	28.0		
ATPγS	8.4	10.6 (d)	22.0 (sd)	−35.0 (d)	19.6	29.0	~5.3	1.4
MgATPγS	8.4	10.6 (d)	20.6 (sd)	−36.8 (d)	15.5	25.4	<4.2	>1.1

[a] s, singlet; d, doublet; t, triplet; sd, split doublet. [b] A and B refer to the diastereoisomers as defined by Eckstein and Goody (1976). [c] 0.5 Hz/point can introduce a maximum uncertainty of 1.0 Hz in each coupling constant listed.

TABLE II: Change in Chemical Shifts and Coupling Constants upon Sulfur Substitution at pH ~8.

Compounds	Δδ (ppm)	Charge on phosphate	Hz $\Delta J_{\alpha\beta}$	$\Delta J_{\beta\gamma}$
ATP-ATPγS	(γ) 41.0	2	~0	9.0
	(γ) >49.5	1		
ATP-ATPβS	(β) 52.1	1	6.5	6.5
ATP-ATPαS	(α) 54.3	1	7.5	~0
ADP-ADPβS	(β) 39.2	2	9.5	
ADP-ADPαS	(α) 51.6	1	8.6	
AMP-AMPS	(α) 39.4	2		
	(α) >48.0	1		
P_i-SP_i	33.7	2		
	44.7	1		

phosphates and thiophosphates are summarized in Table II. (3) Sulfur substitution on any one phosphate of ATP or of ADP enhances the sensitivity of the chemical shift of that phosphate to magnesium binding.

Effect of Magnesium on the NMR Parameters of the Thio Analogues. Magnesium binding to ATP causes a downfield chemical shift change of ~2 ppm in the β-P resonance (Cohn and Hughes, 1962). Sulfur substitution on the β-P increases the effect of magnesium binding on the β-P resonance to ~6 ppm. Sulfur substitution on the α-P also enhances the effect of magnesium binding on the α-P resonance about tenfold from ~0.2 ppm to 2 ppm downfield but does not affect the β-P sensitivity to magnesium binding. For ATPγS again there is an enhancement of the magnesium binding effect on the γ-P resonance from ~0.5 ppm to 1.8 ppm (Figures 1D and 1E and Table I); for this analogue, the large shift of the β-P resonance observed for ATP, >2 ppm, is actually reduced to 1.4 ppm.

No generalizations can be drawn concerning the effect of sulfur substitution on the coupling constants J_{PP} and J_{PS} in the magnesium complexes of the thiophosphate analogues since no simple pattern emerged for the αS, βS, or γS ATPs. As with ATP, for ATPγS both $J_{\alpha\beta}$ and $J_{\beta\gamma}$ decrease about 4–5 Hz upon binding magnesium. On the other hand, in ATPβS, upon chelation neither $J_{\alpha\beta}$ nor $J_{\beta\gamma}$ change significantly (either diastereoisomer). Yet another pattern is observed in the magnesium complex of ATPαS where only $J_{\beta\gamma}$ decreases 5 Hz and $J_{\alpha\beta}$ decreases much less.

Comparison of pH Effects on the Chemical Shifts of Phosphate and Thiophosphate Compounds. The pH titration of orthophosphate is compared with thiophosphate in Figure 2A and of AMP with AMPS in Figure 2B. Not only is the sense of the chemical shift change opposite upon protonation of the normal vs. thiophosphates, but the magnitude of the change in shift between mono- and dianion is considerably greater for the thiophosphates. Protonation of the terminal phosphate of the adenine nucleotides ADP and ATP results in an upfield shift for the terminal phosphate resonance and a small upfield shift for the adjacent phosphate (Cohn and Hughes, 1960; Tran-Dinh et al., 1975). On the other hand, protonation of the terminal thiophosphate of ADPβS and ATPγS, as for inorganic thiophosphate and AMPS, results in a downfield shift in the terminal thiophosphate resonance. Not only is the magnitude of the change in chemical shift between doubly charged and singly charged terminal phosphates increased upon sulfur substitution but a similar increase is observed for any other internal thiophosphate on the molecule. For example, the α-P resonance of ATPαS exhibits a 1 ppm downfield shift upon protonation of its terminal phosphate while the α-P resonance of ATP is barely sensitive to protonation of its terminal phosphate.

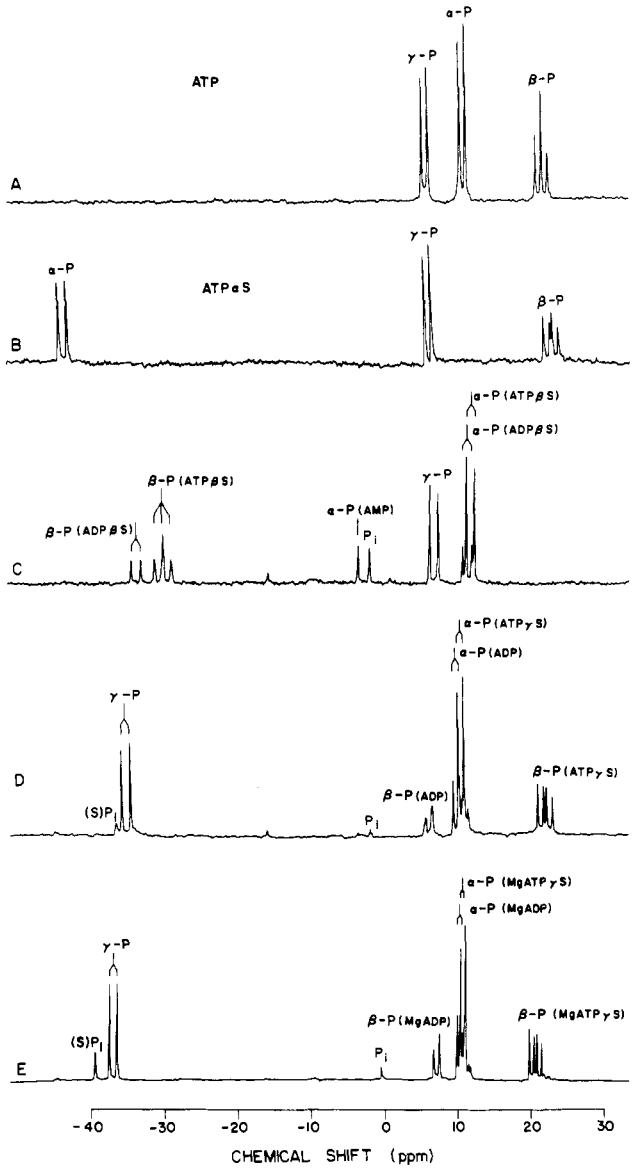

FIGURE 1: (A) ATP, 25 mM (K^+ salt), 2 mM EDTA, pH 11.0, T = 20 °C. NMR parameters: pulse delay, 2 s, 2000 transients. (B) ATPαS, 3.9 mM, diastereoisomer A (NH_4^+ salt), pH 8.0, T = 20 °C. NMR parameters: pulse delay, 1 s, 17 450 transients. (C) ATPβS, diasteroisomer A (K^+ salt), 2 mM EDTA, pH 7.3, T = 20 °C. The sample contained 16.2 mM total adenosine including ATPβS, ADPβS, and AMP, the latter two species arising from the acid hydrolysis of ATPβS. NMR parameters: pulse delay, 1 s, 4400 transients. (D) ATPγS, 14.5 mM (Li^+ salt), 5.5 mM ADP, 2 mM EDTA, pH 7.9, T = 16 °C. NMR parameters: pulse delay, 4 s, 8645 transients. (E) ATPγS, 10.2 mM (Li^+ salt), 4.0 mM ADP, 30 mM $Mg(NO_3)_2$, pH 5.5, T = 15 °C. NMR parameters: pulse delay, 1 s, 15 865 transients. Spectra A through E were obtained at 24.3 MHz.

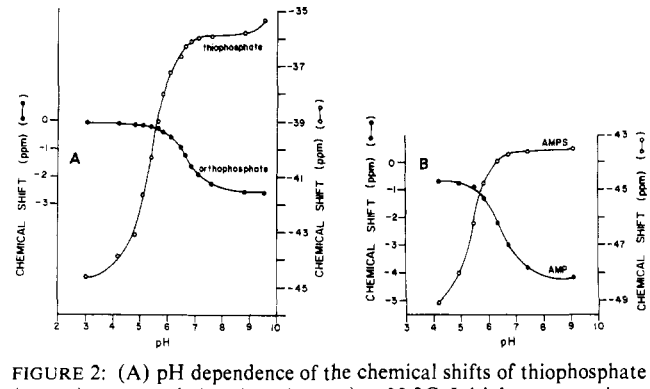

FIGURE 2: (A) pH dependence of the chemical shifts of thiophosphate (O—O) vs. normal phosphate (●—●) at 20 °C. Initial concentrations: 50 mM Na_3SPO_3, 50 mM NaH_2PO_4, 20% D_2O. (B) pH dependence of the chemical shifts of AMPS (O—O) vs. AMP (●—●) at 20 °C. Concentrations: 23 mM AMPS, 22 mM AMP, 1 mM EDTA, 20% D_2O.

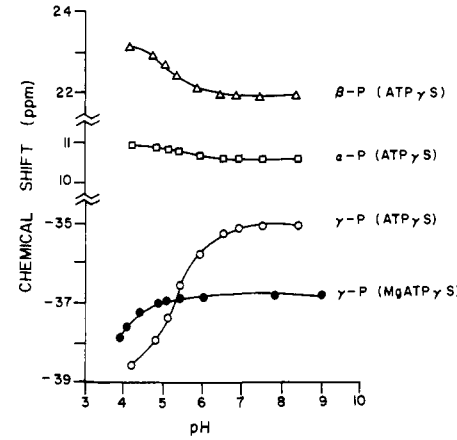

FIGURE 3: pH dependence of the chemical shifts of the α (□—□), β (△—△), and γ (O—O) phosphate resonances at ATPγS and the γ (●—●) phosphate resonance of MgATPγS at 20 °C. ATPγS, 25 mM, 2 mM EDTA, 20% D_2O (O—O). ATPγS, 20 mM, 30 mM $Mg(NO_3)_2$, 2 mM EDTA, 17% D_2O (●—●).

The effect of pH variation between pH 4 and pH 8.5 on each P resonance of ATPγS is shown in Figure 3. In addition to a 4 ppm downfield shift of the γ-P upon protonation, a second-order effect on the chemical shift of the adjacent phosphate (1 ppm upfield) is of the same magnitude and direction as for ATP, but the inflection point has been lowered to pH 5.3 consonant with the lower pK_a of ATPγS (cf. Figure 3). Chelation of ATPγS with magnesium shifts the pK_a but does not affect the direction of chemical shift change upon protonation as shown in Figure 3.

When sulfur is substituted on the β position in ATPβS the terminal phosphate resonance is shifted upfield upon protonation as with ATP. On the other hand, the β-P which has the substituted sulfur, exhibits a small downfield shift, 1 ppm, upon protonation of the γ-phosphate group. Additionally, the α-P resonance of ATPαS exhibits a 1 ppm downfield shift upon protonation of its γ-phosphate group while the β-P and γ-P resonances shift upfield. Thus, protonation of the terminal dianionic phosphate groups always induces a downfield shift on the ^{31}P resonance of that phosphate in the polyphosphate chain which has a sulfur substituting for a nonbridge oxygen.

Effect of Sulfur Substitution on pK_a. Not unexpectedly the apparent second pK_a on the terminal phosphate as determined by the change in chemical shift upon protonation is sensitive to sulfur substitution. The pK_as of ATPγS, ADPβS, AMPS, thiophosphate, and MgATPγS are at least 1 pH unit below the pK_a of the parent compounds, ATP, ADP, AMP, P_i, and MgATP, respectively. Sulfur substitution on the β-phosphate of ATP has a small second-order effect on the second pK_a of the γ-phosphate group. The pK_a data are summarized in Table I.

Chemical-Shift Differences between the Diastereoisomers of ADPαS and ATPβS and Their Magnesium Complexes. ^{31}P chemical shifts of adenine nucleotide phosphates are highly dependent on nucleotide concentration, pH, temperature, and ionic strength so that the precision of chemical-shift differences between spectra taken under even slightly different conditions is questionable. Therefore to ensure identical conditions,

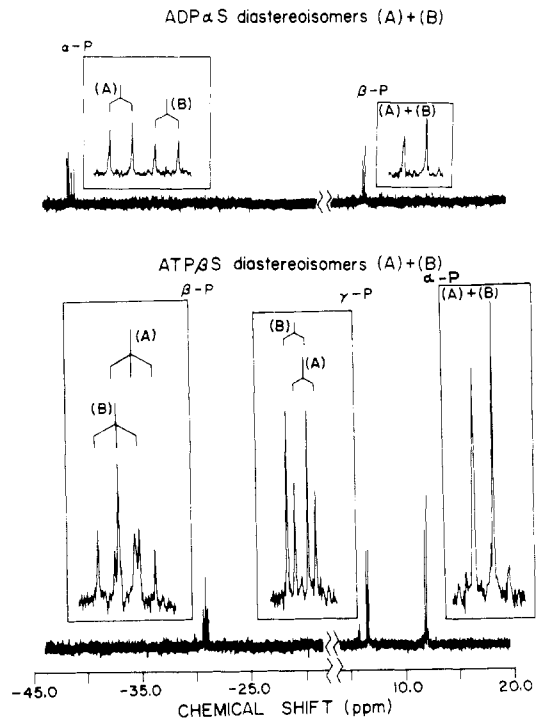

FIGURE 4: Spectra of the mixed diastereoisomers, forms A and B, recorded at 145.7 MHz. Upper spectra: ADPαS (A + B), 2 mM, 0.5 mM EDTA, pH 9.2, 13 °C. Lower spectra: ATPβS (A + B), 5 mM, 6 mM EDTA, pH 7.9, 13 °C. These spectra were obtained on 2.0-mL samples in 10-mm tubes. NMR parameters: acquisition time, 1.34 s; pulse delay, 6 s; pulse width, 45°; spectral width, 12 000 Hz; 157 transients (ADPαS); 199 transients (ATPβS). The inserts are the spectra of the α-, β-, and γ-phosphate resonances, as labeled, recorded with the horizontal scale expanded tenfold.

spectral data were obtained from solutions containing mixtures of isomers of ADPαS and of ATPβS, respectively, and are presented in Table I. Differences in chemical shift were readily observed between the diastereoisomers of both ADPαS and ATPβS.

In the case of ADPαS the β-P resonances of the A and B isomers are not resolved at 24.3 MHz but a separation of the α-P resonances is readily observed; that of the A diastereoisomer of ADPαS is 0.4 ppm downfield from that of the B diastereoisomer. The spectrum of the diastereoisomers of ADPαS was also obtained at 145.7 MHz and is presented in Figure 4A. Although the β-P resonances are again not resolved, the spectral width of 12 000 Hz may have been the limiting factor. At 24.3 MHz, in the presence of magnesium, the β-P resonances of the diastereoisomers of ADPαS remain unresolved but the α-P resonance of the A form is 0.3-ppm downfield from the α-P resonance of the B form.

The β-P chemical shift of the A diastereoisomer of ATPβS is 0.14-ppm upfield from that of the B diastereoisomer but the α-P and γ-P resonances of the two diastereoisomers are indistinguishable at 24.3 MHz. The spectra of the mixed diastereoisomers of ATPβS were also obtained at 145.7 MHz and, as shown in Figure 4B, the γ-P resonances were resolved and separated by 0.045 ppm with the resonance from the A form upfield from that of the B. Again the β-P resonances were readily resolved and the α-P resonances appeared as a single doublet. The limiting factor in resolving the α-P resonances may have been the 12 000-Hz spectral width.

In MgATPβS, the β-P resonance of the A diastereoisomer is 0.4-ppm downfield from that of the B diastereoisomer (cf. Table I). Thus the enhanced sensitivity of the β-P resonance of ATPβS to magnesium binding is even greater for the A form (6.4 ppm) than for the B form (5.9 ppm) (cf. Table I). The γ-P resonance of the magnesium complex of the A diastereoisomer is 0.13 ppm downfield from that of the B diastereoisomer (cf. Table I). On the other hand, in the absence of magnesium, the A isomer is upfield by 0.045 ppm from that of the B diastereoisomer (Figure 4B). Thus upon binding magnesium, the γ-P resonance of the A form moves 0.18 ppm further downfield than that of the B form. In the presence of saturating amounts of magnesium, the α-P resonances of the two diastereoisomers of ATPβS remain unresolved at 24.3 MHz, but are resolved at 145.7 MHz. At the higher frequency a separation of 0.11 ppm between the α-P resonances of the two diastereoisomers was observed with the A form resonance downfield from that of the B form. Thus it appears that the chemical shifts of all three phosphates of the A form of ATPβS are more sensitive to magnesium binding than those of the B form. It may be possible in future double resonance NMR experiments to determine the absolute configuration of the A and B forms.

Discussion

The difference in pK_a of the thio analogues can be utilized in elucidating the nature of the enzyme–substrate complex in those cases where the pK_a of the phosphate group of the substrate apparently changes upon binding to the enzyme. The complex of MgADP with arginine kinase (Nageswara Rao and Cohn, 1977) exemplifies such a case. Not only did the enzyme complex have a pK_a about 1.5 pH units higher than MgADP but the chemical-shift change between protonated and nonprotonated form reversed sense. Since MgADPβS has a pK_a 1.6 pH units lower than MgADP, an unchanged pK_a of the thio analogue–enzyme complex would prove the tentative conclusion that the pK_a of the enzyme bound complex represents the pK_a of an amino acid residue at or near the active site, rather than a change in the pK_a of the β-phosphate group of bound substrate.

Substitution of sulfur for a nonbridge oxygen in phosphate groups increases the sensitivity of the ^{31}P chemical shifts to protonation generally and to magnesium binding in nucleotides. Therefore the thio analogues should prove to be superior probes for determining the ionization state or the extent of magnesium complexation in various systems. A comparison of the available data on the effect of protonation and magnesium binding on the nucleotides and their analogues has reluctantly led us to the conclusion that the magnitude of the change in chemical shifts and coupling constants of ^{31}P resonances upon metal chelation cannot be used to specify the metal binding sites on a polyphosphate chain. The considerations which led to this lamentable conclusion follow. In the case of protonation of the adenine nucleotides, as well in the thio analogues reported in this investigation, in the pH range 5–8 where the site of protonation is known to be the terminal phosphate group, the change in chemical shift upon protonation is by far largest for the phosphate group being titrated. However, for the bridge methylene or bridge imido analogues (Yount, 1975), where again the terminal phosphate is known to be protonated, this is not the case. For the analogues with the bridge oxygen between the terminal phosphate and the adjacent phosphate substituted by a methylene group (Myers, 1967), or an imido group (Tran-Dinh and Roux, 1977), the chemical shift change is much greater on the ^{31}P resonance of the adjacent phosphate than on the terminal phosphate resonance. For example, for deoxy-ATP (β–γ bridge CH_2), the γ-P resonance shifts 1.4 ppm downfield upon protonation of the γ-P group but the β-P resonance shifts 2.7 ppm upfield (Myers, 1967) and for ATP (β–γ bridge imido), the corresponding changes for the γ-P and β-P resonances are 1.43 and 4.01 ppm

upfield, respectively (Tran-Dinh and Roux, 1977). The state of the theory of chemical shifts of ^{31}P resonances at present is inadequate to explain this behavior. Similarly, no adequate explanation is available for the change in the direction of the shift of the terminal phosphate resonances upon protonation of the thio analogues compared with all ^{31}P resonances in monophosphates and polyphosphates. As pointed out by Van Wazer and Letcher (1967), "the variations in the magnetic shielding of the ^{31}P nucleus result from an electronic tug of war about the ^{31}P nucleus from several directions; whereas, for ^{1}H and ^{19}F, the tug is usually all on one side."

From the foregoing discussion it may be concluded that the protonation of a given phosphate group in a polyphosphate chain does not guarantee that the magnitude of the chemical shift change will be greatest for the ^{31}P resonance of that phosphate. Since the magnitude of the chemical-shift change need not be related to the site of binding of a proton, there is no compelling reason for expecting the magnitude of the chemical shift change to be related to the site of magnesium binding. Nevertheless many investigators have done so. In fact for MgATP, based on essentially the same ^{31}P NMR data, Cohn and Hughes (1962) concluded that magnesium was bound to β- and γ-P, Kuntz and Swift (1973) concluded that magnesium was bound to α-, β-, and γ-P and Tran-Dinh et al. (1975) concluded that magnesium was bound to β-P only. As already pointed out (Nageswara Rao and Cohn, 1977) in spite of the small change in chemical shift, the binding of magnesium to the terminal phosphate group of ATP or ADP is unequivocally established independent of NMR data by the change in pK_a indicating competition with the protons on the terminal phosphate. The higher binding constant for magnesium with the nucleoside di- and triphosphates relative to nucleoside monophosphates makes it most likely that magnesium is bound to more than one phosphate group. All previous investigators have interpreted the large shift, 2–3 ppm, on the β-P resonance between ATP and MgATP to establish the existence of a magnesium ligand to the β-phosphate to ATP. To base this conclusion on NMR shift data is questionable in view of the fact that protonation on the γ-phosphate and not on the β-phosphate can lead to a change of 4 ppm in the β-P chemical shift as in AMP-PNP. The chemical-shift data also cannot be used to define the role of α-P in the structure of the magnesium chelate of ATP since, upon binding magnesium, the α-P exhibits a very small shift, ~0.3 ppm, of the same order as that of the γ-P of ATP at high pH. However, in the ATPαS complex with magnesium, the α-P resonance is shifted 2 ppm. Is it then valid to conclude that magnesium is liganded to α-P as well as the β- and γ-P? Probably not, since the chemical shift of an internal thiophosphate has enhanced sensitivity to perturbation of the environment. For example, the β-P resonance of ATPβS is 3–4 ppm more sensitive to magnesium binding than that of ATP (Table I). Thus the chemical-shift data are inconclusive. Nevertheless, the shifts observed upon chelation with magnesium can be used without reservation to determine whether and how much ADP or ATP is magnesium bound. Also a change in the chemical shift of magnesium nucleotides remains an excellent criterion to indicate a change in structure or environment of a given phosphorus atom, e.g., the difference between the ^{31}P resonances of MgATP in aqueous solution and bound to enzymes. Again, chemical-shift data cannot be used to determine the site(s) of magnesium binding to enzyme-bound nucleotides as has been attempted for pyruvate kinase (Gupta and Mildvan, 1977).

The ^{31}P chemical shifts are sensitive to geometry, particularly of the P-X-P bond angles (Gorenstein et al., 1976). In the case of the diastereoisomers of ATPαS, ATPβS, and ADPαS, there is apparently sufficient difference in geometry so that small differences in chemical shifts are observable between the A and B forms. The differences for the A and B forms of ATPαS have recently been reported (Sheu and Frey, 1977). In the case of ATPβS, the differences between the diastereoisomers are enhanced in the magnesium complex where the geometric differences are exaggerated, although no such enhancement was observed for ADPαS. Future NMR experiments may succeed in establishing the absolute configuration of the A and B forms which would aid in delineating the active site structures of the enzymes which show a preference for one form or the other.

Acknowledgment

We thank Mrs. A. Hu for preparation of carp adenylate kinase.

References

Casey, R. P., Njus, D., Radda, G. K., and Sehr, P. A. (1977), *Biochemistry 16,* 972–977.

Cohn, M., and Hughes, T. R., Jr. (1960), *J. Biol. Chem. 235,* 3250–3253.

Cohn, M., and Hughes, T. R., Jr. (1962), *J. Biol. Chem. 237,* 176–181.

Eckstein, F., and Goody, R. S. (1976), *Biochemistry 15,* 1685–1691.

Feldman, K., and Hull, W. E. (1977), *Proc. Natl. Acad. Sci. U.S.A. 74,* 856–860.

Gorenstein, D. G., Findlay, J. B., Momii, R. K., Luxon, B. A., and Kar, D. (1976), *Biochemistry 15,* 3796–3803.

Gupta, R. K., and Mildvan, A. S. (1977), *J. Biol. Chem. 252,* 5967–5976.

Kuntz, G. P. P., and Swift, T. J. (1973), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 32,* 546.

Moon, R. B., and Richards, J. H. (1973), *J. Biol. Chem. 248,* 7276–7278.

Myers, T. C. (1967), in ^{31}P Nuclear Magnetic Resonance, Crutchfield, M. M., Dungan, C. H., Letcher, J. H., Mark, V., and Van Wazer, J. R., Ed., New York, N.Y., Interscience, p 314.

Nageswara Rao, B. D., and Cohn, M. (1977), *J. Biol. Chem. 252,* 3344–3350.

Navon, G., Ogawa, S., Shulman, R. G., and Yamane, T. (1977), *Proc. Natl. Acad. Sci. U.S.A. 74,* 888–891.

Noda, L., Schultz, G. E., and Von Zabern, I. (1975), *Eur. J. Biochem. 51,* 229–235.

Randerath, K., and Randerath, E. (1964), *J. Chromatogr. 16,* 111–125.

Sheu, K. F. R., and Frey, P. A. (1977), *J. Biol. Chem. 252,* 4445–4448.

Tran-Dinh, S., and Roux, M. (1977), *Eur. J. Biochem. 76,* 245–249.

Tran-Dinh, S., Roux, M., and Ellenberger, M. (1975), *Nucleic Acids Res. 2,* 1101–1110.

Van Wazer, J. R., and Letcher, J. H. (1967), in ^{31}P Nuclear Magnetic Resonance, Crutchfield, M. M., Dungan, C. H., Letcher, J. H., Mark, V., and Van Wazer, J. R., Ed., New York, N.Y., Interscience, p 175.

Yount, R. (1975), *Adv. Enzymol. 43,* 1–56.