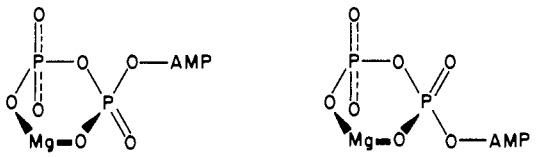
Stability Constants of Mg^{2+} and Cd^{2+} Complexes of Adenine Nucleotides and Thionucleotides and Rate Constants for Formation and Dissociation of MgATP and MgADP†

Vincent L. Pecoraro,‡ Jeffrey D. Hermes, and W. W. Cleland*

ABSTRACT: Stability constants for the Mg^{2+} and Cd^{2+} complexes of ATP, ADP, ATPαS, ATPβS, and ADPαS have been determined at 30 °C and μ = 0.1 M by ^{31}P NMR. Besides being of the utmost importance for determining species distributions for enzymatic studies, these constants allow an estimation of the preference of Cd^{2+} for sulfur vs. oxygen coordination in phosphorothioate complexes. Stability constants for Mg^{2+} complexes decrease when sulfur replaces oxygen (log K: ADP, 4.11; ADPαS, 3.66; ATP, 4.70; ATPαS, 4.47; ATPβS, 4.04) because of (a) a statistical factor resulting from the loss of one potential phosphate oxygen ligand and (b) either an alteration in the charge distribution between oxygen and sulfur or destabilization of the chelate ring structure by loss of an internal hydrogen bond between an oxygen of coordinated phosphate and metal-bound water. Cd^{2+} complexes with sulfur-substituted nucleotides are more stable than those without sulfur (log K: ADP, 3.58; ADPαS, 4.95; ATP, 4.36; ATPαS, 4.42; ATPβS, 5.44) because of the preferential binding of Cd^{2+} to sulfur rather than oxygen, which we estimate to be ~60 in CdADPαS and CdATPβS. The proportion of tridentate coordination is estimated to be 50–60% in MgATP and MgATPβS, ~27% in MgATPαS, ~16% in CdATP or CdATPβS, but ~75% in CdATPαS. By analysis of the data of Jaffe and Cohn [Jaffe, E. K., & Cohn, M. (1979) *J. Biol. Chem. 254*, 10839], we conclude that the preference for oxygen over sulfur coordination to ATPβS is 31 000 for Mg^{2+}, 3100–3900 for Ca^{2+}, and 158–193 for Mn^{2+}. Proton NMR demonstrates that bidentate Cd^{2+} complexes form intramolecular chelates with the N-7 of adenine while Mg^{2+} nucleotides and the tridenate CdATPαS do not. An analysis of the ^{31}P NMR line widths shows that the rate constants for dissociation of MgADP and MgATP are both 7000 s^{-1} while the association rate constants are 7×10^7 and 4×10^8 M^{-1} s^{-1}, respectively. The observed dependence of the line width on nucleotide concentration is best explained by a base-stacking model at nucleotide concentrations above 5 mM.

Metal complexes of adenine nucleotides are involved in many biochemical reactions, and thus, the chemistry of the complexes which ATP, ADP, and AMP form with metal ions has been extensively studied to determine stability constants (Smith & Alberty, 1956; Martell & Swarzenbach, 1956; O'Sullivan & Perrin, 1964; Phillips, 1966; Kahn & Martell, 1967; Frey & Stuehr, 1972; Adolfsen & Moudrianakis, 1978; Gerlt et al., 1983), the mode of metal ion coordination (Cohn & Hughes, 1962; Huang & Tsai, 1982; Scheller & Sigel, 1983a,b), and the stereochemistry of the coordination (Cornelius et al., 1977; Merritt et al., 1978; Dunaway-Mariano & Cleland, 1980a,b). Experiments with inert cobalt and chromium complexes have demonstrated that Λ and Δ screw sense diastereomers of bidentate complexes can exist in solution[1]



Λ-MgATP Δ-MgATP

and that enzymes show a distinct preference for one of the two isomers (Dunaway-Mariano & Cleland, 1980b). An alternate approach for determining the screw sense specificity of enzymes is to use as substrates in the enzymatic reation nucleoside phosphorothioates, which are structural analogues of nucleotides in which a sulfur atom replaces one of the oxygens bonded to phosphorus (Eckstein, 1975; Cohn, 1982). If the sulfur substitution is on an internal phosphate, the phosphorus becomes chiral instead of prochiral. Jaffe & Cohn (1978b) demonstrated that Mg^{2+} coordinates predominantly to phosphate oxygens while Cd^{2+} prefers sulfur ligation. Thus, with a given isomer of a chiral nucleoside phosphorothioate one can generate either screw sense isomer by proper choice of the divalent metal. Comparison of the kinetic parameters for the two metal ions with the two isomers of the chiral phosphorothioate allows one to deduce the chemical mechanism and screw sense specificity for a wide variety of enzymatic reactions (Sheu & Frey, 1977; Jaffe & Cohn, 1978b; Raushel et al., 1978; Armstrong et al., 1979; Jaffe & Cohn, 1979).

Although a large amount of effort has been expended on the enzymology associated with nucleoside phosphorothioates, only limited knowledge is available on the solution chemistry of their metals complexes. The ^{31}P NMR chemical shifts for the free nucleotides and various metal complexes (Sheu & Frey, 1977; Jaffe & Cohn, 1978a) as well as the pK values for the external thiophosphate analogues (Jaffe & Cohn, 1978a; Gerlt et al., 1983) have been reported. The preferences of different metals for coordination of sulfur or oxygen have been qualitatively deduced, but no direct quantitative measurements have been made.

In this paper we report the stability constants for the Mg^{2+} and Cd^{2+} complexes formed with internal sulfur-containing nucleoside phosphorothioates and the corresponding non-sulfur-containing nucleotides, as well as the pK values for the

† From the Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706. *Received February 7, 1984.* This work was supported by a grant from the National Science Foundation (PCM 8200705). V.L.P. held an NIH Postdoctoral Fellowship (GM 08568).

‡ Present address: Department of Chemistry, University of Michigan, Ann Arbor, MI 48109.

[1] The screw sense nomenclature is that of Cornelius & Cleland (1978). In this system the reference axis is a line through the metal perpendicular to the chelate ring, and the bond from the chelate ring to the rest of the molecule is the skew line defining either a left-hand (Λ) or right-hand (Δ) helix.

0006-2960/84/0423-5262$01.50/0

metal complexes. We can estimate from these data the relative levels of Cd–O and Cd–S coordination, and the fraction of tridentate coordination in CdATPαS[2] can be calculated from the magnitude of the ^{31}P NMR shift. Proton NMR has been used to monitor the involvement of the adenine N-7 atom in coordination to the metal. Correlation of our data with the kinetic constants reported for hexokinase by Jaffe & Cohn (1978b) allows us to determine for this enzyme the degree of nonproductive binding by the wrong screw sense isomers, the effect on V and V/K of sulfur in the noncoordinated as well as the coordinated position, and the ratio of oxygen to sulfur coordination for those other divalent metal ions with which product release is not rate limiting. On the basis of ^{31}P NMR line-width measurements, we also report the rate constants for formation and breakdown of MgATP and MgADP.

Materials and Methods

Materials. ATP, ADP, AMPαS, ADPβS, phosphoenolpyruvate, NADH, pyruvate kinase, and lactate dehydrogenase were from Boehringer. ATPαS (S_P isomer), ATPβS (R_P), and ADPαS (S_P) were synthesized enzymatically by the methods of Eckstein & Goody (1976). Thionucleotides were purified by elution from a DEAE-Sephadex column with a 0.05–1 M gradient of triethylammonium bicarbonate, pH 7.6. Yeast hexokinase, glucose-6-P dehydrogenase, NADP, EDTA, and ion-exchange resins were from Sigma. Magnesium metal (Fischer) was dissolved in 1 N HNO_3 to make a stock solution of Mg^{2+}, while $CdCl_2$ (Baker) was used to prepare a Cd^{2+} stock solution. Eriochrome Black T (Clarkson Labs) was used as indicator in the standardization of metal stock solutions by titration with EDTA by the method of Welcher (1958).

Preparation of Solutions. Aqueous solutions of Na_2ATP or NaADP were made to ~5 mM, and the exact concentrations were determined with enzymatic end point assays (a hexokinase–glucose-6-P dehydrogenase assay with NADP was used for ATP, and a pyruvate kinase–lactate dehydrogenase assay with NADH for ADP, assuming $\epsilon = 6300\ cm^{-1}\ M^{-1}$ at 340 nm for the spectral changes). All measurements were on solutions maintained at 0.1 M ionic strength by addition of KNO_3. The thionucleotides were converted from the triethylammonium form to the K^+ form by making a slurry with Dowex 50-K^+, followed by filtration, and their concentrations were measured spectrally, assuming $\epsilon = 15\,400\ cm^{-1}\ M^{-1}$.

Instrumentation. UV absorbances were measured with a Beckman DU monochromator, a Gilford OD Converter, and a 10-mV recorder. ^{31}P NMR spectra (80.99 MHz) were obtained with a Nicolet NT-200 Fourier transform spectrometer with a 12-mm broad-band probe. Samples were placed in the outer portion of 10-mm coaxial tubes, with a solution of phosphate at pH 7.0 in D_2O in the internal tube used as a lock signal and a standard in the spectrum. A transmitter pulse of 15 μs (60°) with a recovery time of 4.0 s was used. Digital filtering of 1.0 Hz was applied before Fourier transformation of the free induction decay. The temperature was maintained between 30 and 32 °C with air cooling of the bilevel proton decoupled sample. The terminal phosphorus peak was always used in titrations as the value for the chemical shift in data analysis. In stability constant titrations an aliquot of metal was added to the sample and the pH adjusted to the original hydrogen ion concentration with KOH. The concentrations of ligand and metal were corrected for this dilution. Because of the intermediate exchange rates observed for solutions containing MgATP and HATP, MgADP and HADP, and MgATPαS and HATPαS, the midpoint of the broadened phosphorus resonance was used in data analysis.

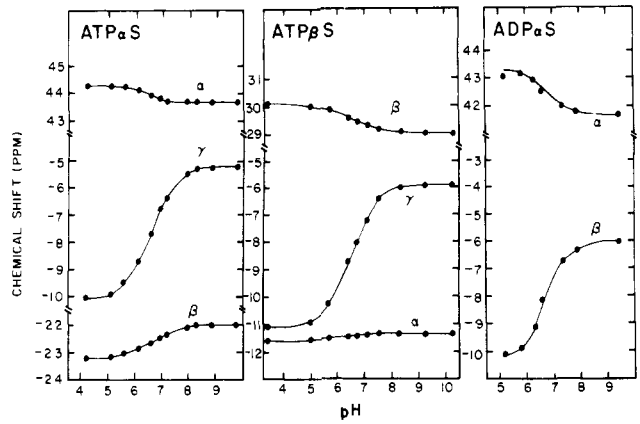


FIGURE 1: ^{31}P NMR chemical shifts of 5 mM thionucleotides when titrated with acid at 30 °C in 0.1 M KNO_3.

Proton NMR spectra to investigate the interactions of metal ions with the adenine ring were obtained at 270 Mhz on a Bruker WH-270 instrument interfaced to a Nicolet 1180 data acquisition system. Acquisition parameters included a 90° transmitter pulse of 9 μs, sweep width of 3000 hz, and a recovery time of 1.36 s. The ADP, ATP, Cd^{2+}, and Mg^{2+} stock solutions were lyophilized 3 times from D_2O and then dissolved in D_2O (100.0 atom % D; Aldrich) which was very low in paramagnetic impurities (τ_1 = 23 s without degassing). Proton chemical shifts were referenced to a 3-(trimethylsilyl)propanesulfonic acid internal standard (δ = 0.00).

Data Analysis. Equation 1 relates the observed chemical

$$\Delta_{obsd} = (\Delta_1 - \Delta_{obsd})K_a/[H^+] + \Delta_2 \quad (1)$$

shift, Δ_{obsd}, to the acid dissociation constant for a ligand or metal–ligand complex. This equation is a modification of that used by Schwarzenbach & Schwarzenbach (1963) for analysis of visible spectral changes. In eq 1, Δ_1 and Δ_2 represent chemical shifts for the fully ionized and monoprotonated forms of the ligand. A least-squares fit of Δ_{obsd} vs. $(\Delta_1 - \Delta_{obsd})/[H^+]$ allows calculation of K_a and Δ_2 (Δ_1 is readily measured independently).

Stability constants for reaction 2 can be calculated from eq 3:

$$M + L \rightleftharpoons ML \quad (2)$$

$$K_{ML} = [ML](1 + [H^+]/K_1)/\{[[M_t] - [ML] \times (1 + [H^+]/K_2)][[L_t] - [ML](1 + [H^+]/K_2)]\} \quad (3)$$

where $[L_t]$ and $[M_t]$ are the total analytical concentrations of ligand and metal in all forms in solution and

$$K_1 = [L][H^+]/[HL] \quad (4)$$

$$K_2 = [ML][H^+]/[HML] \quad (5)$$

$$[ML] = [L_t](\Delta_{obsd} - \Delta_1)/[(\Delta_2 - \Delta_1)(1 + [H^+]/K_2)] \quad (6)$$

with Δ_{obsd} being the observed chemical shift and Δ_1 and Δ_2 the limiting chemical shifts at constant pH in the absence of metal and at saturating metal concentrations, respectively. The stability constant for the reaction of metal with the monoprotonated form of the nucleotide is then given by

$$K_{HML} = K_{ML}K_1/K_2 \quad (7)$$

[2] Abbreviations: EDTA, ethylenediaminetetraacetic acid; ATPαS, ATPβS, ADPαS, ADPβS, and AMPαS, adenine nucleotides substituted with sulfur on the indicated phosphate. The prefixes Mg or Cd (as in CdATPαS) indicate a complex of Mg^{2+} or Cd^{2+} with the indicated nucleotide. The letter H indicates that the terminal phosphate is protonated (as in MgHADP).

Table I: pK Values and ^{31}P NMR Shifts for Adenine Nucleotides and Their Mg^{2+} and Cd^{2+} Complexes

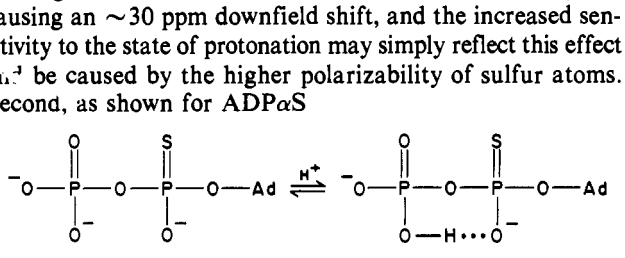
	pK		chemical shift[a] fully ionized			chemical shift[a] monoprotonated[c]		
nucleotide	this work[b]	previous work	P_α	P_β	P_γ	P_α	P_β	P_γ
ATP	6.63 ± 0.04	6.7,[d] 6.95,[e] 6.56[f]	−10.63 (d)	−21.37 (t)	−5.70 (d)	−10.87 (d)	−22.55 (t)	−10.28 (d)
ADP	6.66 ± 0.01	6.8,[d] 6.68,[e] 6.40[f]	−10.18 (d)	−5.86 (d)		−10.77 (d)	−10.19 (d)	
ATPαS	6.65 ± 0.01		43.52 (d)	−22.05 (m)	−5.28 (d)	44.06 (d)	−23.27 (m)	−10.07 (d)
ATPβS	6.64 ± 0.10	6.5[d]	−11.55 (d)	28.99 (t)	−6.10 (d)	−11.77 (d)	30.24 (t)	−11.26 (d)
ATPγS		5.3,[d] 5.8[g]	−10.6 (d)[d]	−22.0 (m)[d]	35.0 (d)[d]			
ADPαS	6.77 ± 0.08		41.64 (d)	−6.05 (d)		42.97 (d)	−10.48 (d)	
ADPβS		5.2[d]	−11.1 (d)[d]	33.9 (d)[d]				
MgATP	4.72 ± 0.05	5.3,[d] 5.0,[e] 4.55[f]	−10.95 (d)	−19.28 (t)	−5.82 (d)	−11.44 (d)	−22.13 (t)	−10.80 (d)
MgADP	5.46 ± 0.06	5.12,[e] 4.91[f]	−9.77 (d)	−5.72 (d)		−10.93 (d)	−10.65 (d)	
MgATPαS	5.12 ± 0.03		45.16 (d)	−19.87 (m)	−5.64 (d)	44.66 (d)	−23.42 (m)	−10.31 (d)
MgATPβS	5.05 ± 0.04		−11.34 (d)	31.91 (t)	−5.78 (d)	−12.03 (d)	30.11 (t)	−11.18 (d)
MgADPαS	5.27 ± 0.03		44.02 (d)	−6.55 (d)		44.14 (d)	−11.51 (d)	
CdATP	4.15 ± 0.16		−10.30 (d)	−16.87 (t)	−4.23 (d)	−10.32 (d)	−19.97 (t)	−7.38 (d)
CdADP	4.82 ± 0.21		−6.98 (d)	−4.00 (d)		−8.12 (d)	−7.53 (d)	
CdATPαS	4.19 ± 0.04		40.75 (d)	−19.11 (m)	−3.36 (d)	39.85 (d)	−21.52 (m)	−8.27 (d)
CdATPβS	4.18 ± 0.17		−11.31 (d)	24.30 (t)	−6.52 (d)	−11.98 (d)	26.95 (t)	−11.71 (d)
CdADPαS	4.00 ± 0.15		36.75 (d)	−4.92 (d)		38.40 (d)	−9.1 (d)	

[a] ^{31}P chemical shifts are given at the center of multiplets in ppm from 85% H_3PO_4. Upfield shifts are negative. (d) Doublet; (t) triplet; (m) multiplet. All spectra were broad-band proton decoupled. [b] 30 °C, μ = 0.1 M (KNO_3). [c] Values for the complexes are calculated from eq 1. [d] Determined by ^{31}P NMR at 18 °C by Jaffe & Cohn (1978a). [e] Determined by potentiometric titration at 25 °C, μ = 0.2 M, by Smith & Alberty (1956). [f] Determined by potentiometric titration at 25 °C, μ = 0.1 M (Martell & Smith, 1976). [g] Determined by ^{17}O NMR at 30 °C by Gerlt et al. (1983).

Results and Discussion

Ligand pK Values. The pH titration curves for ATPαS, ATPβS, and ADPαS are shown in Figure 1. The pK values for ATP, ADP, ATPαS, ATPβS, and ADPαS are in Table I along with the ^{31}P NMR chemical shifts for the fully ionized and monoprotonated forms of the nucleotides. It is evident that replacement of an oxygen by sulfur on an internal phosphorus atom has little effect on the ligand pK values. In contrast, the pK for the terminally substituted phosphorothioate, ATPγS, is 1.4 pH units less than that of ATP, and the pK for ADPβS is perturbed downward by 1.6 pH units (Jaffe & Cohn, 1978a; Gerlt et al., 1983). This behavior is not surprising since the protonation occurs on the terminal phosphate.

As shown in Figure 1, the ^{31}P chemical shifts for internal sulfur-substituted phosphates are sensitive to the protonation state of the ligand. Thus, the α-phosphate of ATPβS undergoes less than a 0.25 ppm upfield shift upon protonation of the γ-phosphate, while the α-phosphorus of ATPαS shows greater than a 0.5 ppm downfield shift under similar conditions. This effect is even more pronounced when the α-phosphate of ADP is compared to that of ADPαS (Table I). This sensitivity of the sulfur-containing phosphate group may be the result of two factors. First, sulfur substitution has a much greater effect on ^{31}P NMR shifts than does oxygen, causing an ~30 ppm downfield shift, and the increased sensitivity to the state of protonation may simply reflect this effect and be caused by the higher polarizability of sulfur atoms. Second, as shown for ADPαS



on protonation an internal hydrogen bond can form between the α- and β-phosphate oxygens, thus increasing the P–S double bond character and causing a downfield shift.

We should note that the constants reported in Tables I and II were calculated for the S_P isomers of ADPαS and ATPαS and the R_P isomer of ATPβS. Because these complexes are diastereomers, it is possible that the constants may be slightly different for the opposite isomers, but we believe these differences will be very small.

Table II: Stability Constants for Complexes of Adenine Nucleotides with Mg^{2+} and Cd^{2+}

	log K[a]	
complex	this work[b]	previous work
MgATP	4.70 ± 0.12	4.06,[c] 3.47,[d] 4.84[e]
MgADP	4.11 ± 0.16	3.17,[c] 3.00,[d] 3.93[e]
MgATPαS	4.47 ± 0.13	
MgATPβS	4.04 ± 0.15	
MgADPαS	3.66 ± 0.10	
MgHATP	2.79 ± 0.15	2.10,[c] 1.49,[d] 2.92[e]
MgHADP	2.94 ± 0.14	1.67,[c] 1.45,[d] 2.72[e]
MgHATPαS	2.94 ± 0.10	
MgHATPβS	2.45 ± 0.18	
MgHADPαS	2.16 ± 0.12	
CdATP	4.36 ± 0.28	
CdADP	3.58 ± 0.21	
CdATPαS	4.92 ± 0.23	
CdATPβS	5.44 ± 0.13	
CdADPαS	4.95 ± 0.18	
CdHATP	1.88 ± 0.24	
CdHADP	1.74 ± 0.31	
CdHATPαS	2.46 ± 0.07	
CdHATPβS	2.98 ± 0.15	
CdHADPαS	2.18 ± 0.19	

[a] K is the equilibrium constant in M^{-1} for the reaction of ligand and metal ion to give the complex. [b] 30 °C, μ = 0.1 M. [c] Potentiometric titration, 25 °C, μ = 0.1 M (Martell & Smith, 1976). [d] Potentiometric titration, 25 °C, μ = 0.2 M (Alberty & Smith, 1956). [e] Adolfsen & Moudrianakis (1977).

Metal Chelate pK Values. The pK values for the Mg^{2+} and Cd^{2+} complexes of ATP, ADP, ATPαS, ATPβS, and ADPαS are in Table I, along with the ^{31}P NMR chemical shifts for the protonated and unprotonated forms of the complexes. The chemical shifts of the protonated complexes were obtained from least-squares fits to eq 1, since at the low pH required for full protonation of the complexes the weak complexation of the metal by the protonated nucleotides made direct experimental determinations impractical. Two trends are apparent from these pK values. First, the Cd^{2+} complexes are more acidic than the Mg^{2+} ones, and second, the ATP complexes are more acidic than the ADP complexes. We cannot explain why the pK for CdADPαS is so low, since a value of

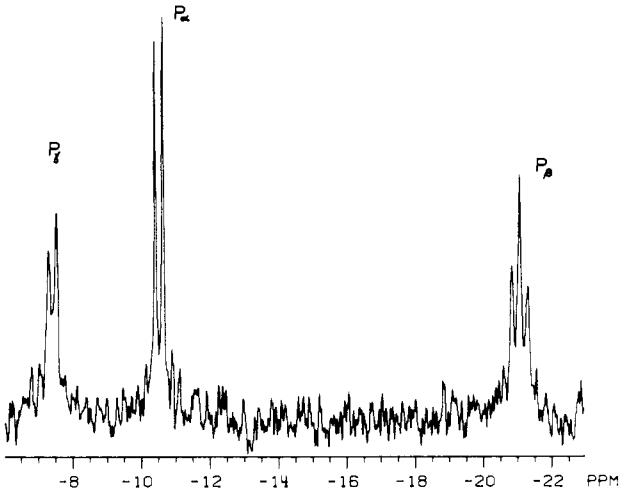


FIGURE 2: ^{31}P NMR spectrum of CdATP at ~50% saturation with metal ion. [ATP] = 3.47 mM, pH 6.35, 30 °C, μ = 0.1 M KNO_3.

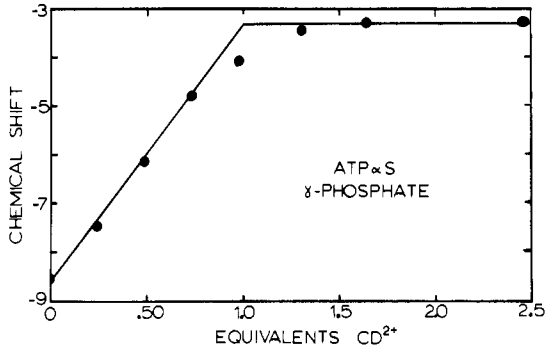


FIGURE 3: Titration of 2.3 mM ATPαS with Cd^{2+}. pH 6.25, 30 °C, μ = 0.1 M KNO_3.

4.5–5.0 would be expected on the basis of the other constants.[3] In any case, it is clear that, under the conditions that one would carry out most enzymological studies, the protonated forms of the nucleotide complexes will be present at very low levels.

Protonation of the metal–nucleotide complexes most likely occurs on one of the terminal phosphate oxygens, since a 5 ppm perturbation of the chemical shift of the terminal phosphate accompanies protonation. Jaffe & Cohn (1978a) report that for phosphate groups with methylene or imido bridges the phosphate being titrated does not always have the largest chemical shift, but all data for oxygen-bridged adenine nucleotides and their thio analogues indicate that the phosphate being protonated shows the largest shift.

Stability Constants. Stability constants for the Mg^{2+} and Cd^{2+} complexes of ATP, ADP, ATPαS, ATPβS, and ADPαS were obtained by titrating a solution of nucleotide with the appropriate metal ion at a pH where the free ligand was predominately protonated. In this way, solutions could be made which were concentrated enough to obtain spectra with good signal/noise in 15–30 min while still allowing a fraction of the total metal ion to remain uncomplexed (Figure 2), which is critical for obtaining accurate values of the stability constants

[3] The pK values for the Cd^{2+} thionucleotide complexes are expected to have the highest uncertainty for two reasons. First, it is difficult to go low enough in pH to define the limiting low pH value because decomposition of the ligand occurs in acidic solution. Second, to ensure that complex formation is complete at low pH, a large excess of metal ion is required, but at higher pH this metal ion precipitates, causing broadening of the NMR peaks. Because both of these problems are worse for Cd^{2+} (solubility products are lower and these complexes are more acidic), the values for cadmium complexes are least well-defined.

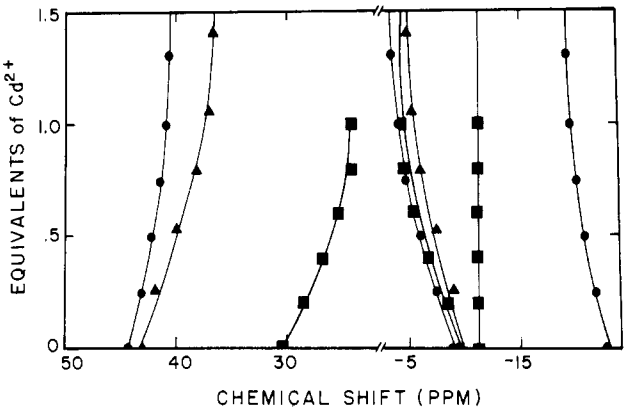


FIGURE 4: Chemical shift variation of 2 mM thionucleotides on addition of Cd^{2+}. ATPαS (●); ATPβS (■); ADPαS (▲). pH 6.2, 30 °C, μ = 0.1 M.

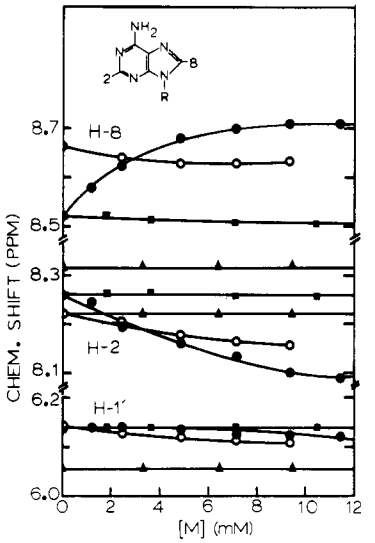


FIGURE 5: Proton NMR titration curves for addition of Cd^{2+} to ATP (●), adenosine (▲), and ATPαS (○) and of Mg^{2+} to ATP (■). H-1′ is the anomeric proton. Nucleotide concentrations ~ 6.5 mM, at pH 5.5, 25 °C.

Table III: Changes in ^{31}P NMR Chemical Shifts on Metal Complex Formation

complex formed	reference form of nucleotide: fully ionized[a] P_α	P_β	P_γ	monoprotonated[b] P_α	P_β	P_γ
MgATP	−0.32	+2.09	−0.12	−0.08	+3.27	+4.46
MgADP	+0.41	+0.14		+1.00	+4.47	
MgADPαS	+2.57	−0.32		+1.19	+4.09	
MgATPαS	+1.64	+2.18	−0.36	+1.10	+3.40	+4.43
MgATPβS	+0.21	+2.92	+0.32	+0.43	+1.67	+5.48
CdATP	+0.32	+2.50	+1.47	+0.57	+3.68	+6.05
CdADP	+3.20	+1.86		+3.79	+6.19	
CdADPαS	−4.89	+1.13		−6.22	+5.56	
CdATPαS	−2.83	+2.94	+1.92	−3.31	+4.16	+6.71
CdATPβS	+0.24	−4.69	−0.42	+0.46	−5.94	+4.74

[a] Chemical shift for metal–nucleotide complex (with a charge of 2– for triphosphates and 1– for diphosphates) minus that for the fully ionized nucleotide. [b] Chemical shift for metal–nucleotide complex (with a charge of 2– for triphosphates and 1– for diphosphates) minus that for the monoprotonated nucleotide.

(Rossotti & Rossotti, 1961). Figure 3 shows how the data are plotted. The vertical intercept gives the chemical shift of the ligand in the absence of metal ion, while the value on the plateau is for the fully formed metal complex. The intersection point of the initial tangent and the plateau gives the metal stoichiometry for the combination (always 1:1 in these studies).

It is important that the curve cut the corner sufficiently to permit accurate determination of stability constants.

The effect of Cd^{2+} complexation on the chemical shifts of the phosphorothioates is shown in Figure 4. As reported by Jaffe & Cohn (1979) complexation of a metal ion with oxygen causes a downfield shift and with sulfur, an upfield shift in the ^{31}P NMR spectrum. Our data thus support the suggestion of Jaffe & Cohn (1979) that Mg^{2+} binds preferentially to phosphate oxygens and Cd^{2+} to sulfur. The presumably bidentate CdATPβS and CdADPαS complexes show an upfield shift of 6.0 ppm for the thiophosphate, while the value is 3.3 ppm for CdATPαS, suggesting that ~55% of this complex is a tridentate chelate (we will estimate this proportion in a different way below)[4] (Table III).

The stability constants for the Mg^{2+} and Cd^{2+} complexes of ATP, ADP, ATPαS, ATPβS, and ADPαS are in Table II. The values for MgATP and MgADP are very similar to those reported by Adolfsen & Moudrianakis (1978), although they are somewhat greater than values from older determinations. While Cd^{2+} forms weaker complexes than Mg^{2+} with ATP and ADP, the opposite is true for the phosphorothioates.[5] While MgATPαS, which is presumably largely a β,γ-bidentate chelate, has nearly the same stability as MgATP, the phosphorothioates which have sulfur on a phosphate coordinated to Mg^{2+} show a marked destabilization. By contrast all of the Cd^{2+} complexes with phosphorothioates are more stable than those with the parent nucleotides.

Table II also includes the stability constants for the reaction of metal ion with the monoprotonated nucleotide to form the protonated metal complex. These values were calculated from eq 8 and are substantially less than the values for the fully ionized nucleotides.

In reversal studies the kinetic parameters for an enzymatic reaction are determined by using both the Mg^{2+} and Cd^{2+} complexes of both diastereomers of a chiral nucleoside phosphorothioate. To obtain accurate values for V_{max}, V/K, and K for each of the alternate substrates, it is necessary to know the proportion of metal bound to the nucleotide, especially for Cd^{2+} where metal to nucleotide ratios above 1.0 often inhibit the enzyme and thus cannot be used. The stability constants and pK values presented above allow accurate determination of the species distribution for ATP, ADP, ATPαS, ATPβS, and ADPαS complexes with Cd^{2+} or Mg^{2+} at any pH above 4.

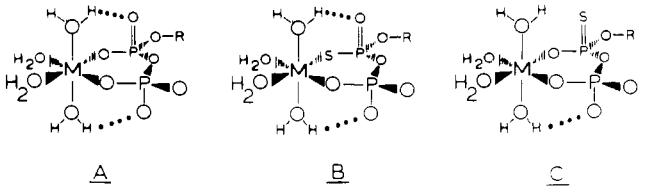


FIGURE 6: Three possible hydrogen bonding conformations of adenine nucleotides. In (A) and (B) two strong internal hydrogen bonds between a metal-bound water and a phosphate oxygen are formed, while in (C) the sulfur does not have a strong interaction with the water proton. Complexes B and C represent the complexes with coordinated and noncoordinated sulfur that will occur in kinetic studies with phosphorothioates. Note that adenosine is pseudoequatorial in all structures.

Involvement of Adenine Nitrogen Atoms in Metal Complexation. Proton NMR spectroscopy was used to monitor the involvement of N-7 of adenosine in coordination to Mg^{2+} or Cd^{2+}. Figure 5 shows how the chemical shifts of H-8, H-2, and the anomeric proton are perturbed on addition of metal ion. Addition of Mg^{2+} to ATP has little effect on the proton spectrum while titration with Cd^{2+} generates a classic binding curve for the change in chemical shift at H-8.[6] Scheller et al. (1981) have proposed that this behavior indicates coordination of N-7 directly to the metal ion, so that a macrochelate forms with two phosphate oxygens and N-7 of adenine in the first coordination sphere of the Cd^{2+} ion. Titration of adenosine with Cd^{2+} caused no change in the chemical shift of H-8, indicating that phosphate coordination is required before N-7 can bind. Most intriguing, however, are the data for CdATPαS. A downfield shift of H-8 indicates metal coordination, but here H-8 moves upfield, as does H-2 and H-1′. Scheller et al. (1981) have shown that upfield shifts are consistent with the isodesmic model of indefinite noncooperative base stacking. Thus, it appears that CdATPαS does not form an N-7 macrochelate complex both because so much of it is an α,β,γ-tridentate chelate and because sulfur substitution for oxygen enhances base stacking of the Cd^{2+} complex.

Ratio of Sulfur and Oxygen Binding in Cd^{2+} Phosphorothioate Complexes. The data in Table II allow calculation of the ratio of sulfur and oxygen binding by Cd^{2+} to phosphorothioates. For CdADPαS, the observed stability constant, K_{obsd}, is the sum of the stability constants for the Cd–O ($K_{\alpha os}$) and Cd–S ($K_{\alpha so}$) bidentate complexes (the three letters in the subscript indicate the phosphate group and the coordinated and noncoordinated atoms of this phosphate group):

$$K_{obsd} = K_{\alpha os} + K_{\alpha so} \quad (8)$$

To obtain a value of $K_{\alpha os}$, we use the following analysis. The log K_{obsd} values for MgADP and MgADPαS differ by 0.45, which corresponds to a ratio of 2.8 between K_{obsd} values. The equations which govern this situation are

for MgADP

$$K_{obsd} = 2K_{\alpha oo} \quad (9)$$

for MgADPαS

$$K_{obsd} = K_{\alpha os} \quad (10)$$

We have omitted $K_{\alpha so}$ from eq 10, since for Mg^{2+} it is much

[4] Jaffe & Cohn (1978) have shown that trends in ^{31}P chemical shifts can be unpredictable, and they warned against their use in determining phosphate involvement in metal coordination. The γ-phosphate of ATP is coordinated to Mg^{2+}, but the observed chemical shift difference between the metal complex and fully deprotonated ATP is 0.12 ppm, which is less than the difference observed for the α-phosphate which is only weakly coordinated (Table II). However, comparison of the chemical shifts for HATP and MgATP indicates that (a) the β- and γ-phosphates which are involved in metal coordination have large chemical shift differences between these species while the largely noncoordinated α-phosphate is little affected and (b) the magnitude of this change is consistent for each metal (~ +4.5 ppm for Mg^{2+} and ~ +6.2 ppm for Cd–O for a terminal phosphate; ~ −6.0 ppm for Cd–S for internal thiophosphates). With this reference system the ^{31}P NMR chemical shift data are consistent and can be used to obtain a quantitative estimate of tridentate coordination for CdATPαS. Additional support for tridentate coordination for this complex comes from the stability constants in Table II where log K for CdATPαS (4.95) is intermediate between the values for CdATPβS (5.44) and CdATP (4.36) and from the more detailed calculation given.

[5] It is not surprising that Mg^{2+} would form a more stable complex with ATP and ADP than Cd^{2+}. The Mg^{2+} ion is a hard Lewis acid preferring to coordinate hard Lewis bases such as oxyanions (Cotton & Wilkenson, 1972), while Cd^{2+} is a soft Lewis acid with high affinity for larger, more polarizable ligands such as sulfur atoms.

[6] The titration curve for H-2 is composed of two equilibria. The first corresponds to Cd^{2+} coordination to phosphates and N-7, while the second saturates with respect to the free metal concentration. The logs of the stability constants for the second process are 3.01 and 4.59 for CdATP and CdATPαS, respectively. We believe this behavior indicates that a second Cd^{2+} ion can be bound to these nucleotides at high free metal concentration.

less than $K_{\alpha os}$ (see below). Note that the availability of two oxygens in the α position of ADP leads to a statistical factor of 2 in eq 9.

These equations predict that the ratio of stability constants for MgADP and MgADPαS will be $2K_{\alpha oo}/K_{\alpha os}$. Since the ratio is 2.8 rather than 2, it is clear that $K_{\alpha oo}$ is greater than $K_{\alpha os}$. This could result from a greater charge density on sulfur than on oxygen in the α position (each oxygen has half a negative charge on the α-phosphate of ADP, but the charge may be unequally distributed in a thionucleotide). Such an altered charge distribution should have an equal effect on the complexes with Mg^{2+} and Cd^{2+}. Another possible contributing factor is the fact that replacement of oxygen with sulfur will prevent formation of a hydrogen bond with metal-bound water (Figure 6). Such hydrogen bonds are thought to stabilize the isomers of the inert CrATP complex so that ring conformers interconvert only slowly (Dunaway-Mariano & Cleland, 1980a), although the pK of water bound to Cr^{3+} is much lower than that of water bound to Mg^{2+} or Cd^{2+}. Because the pK of water bound to Cd^{2+} is about 2 pH units less than for Mg^{2+}, this effect may be larger for that portion of CdADPαS which is oxygen coordinated than for MgADPαS, but we will assume it is the same.

As the result of the above analysis, we assume that $K_{\alpha os}$ for CdADPαS is less than K_{obsd} for CdADP by a factor of 2.8, or equal to $10^{3.13}$. Equation 8 thus yields $10^{4.49}$ for $K_{\alpha so}$. The ratio of Cd–S to Cd–O complexes is given by $K_{\alpha so}/K_{\alpha os}$, which is 65 in this case.

With ATPβS, we must consider the effect of tridentate as well as bidentate coordination. For MgATP

$$K_{obsd} = 2K_{\beta oo}(1 + K_{\alpha oo}) \quad (11)$$

where $K_{\beta oo}$ is the stability constant for formation of a bidentate chelate with a given oxygen at the β position when the other atom attached to the β position is oxygen and $K_{\alpha oo}$ is the equilibrium constant for conversion from bidentate to tridentate coordination with a given oxygen at the α position, with the other atom at the α position being oxygen. If we assume that the tendency to coordinate to the α-phosphate is the same regardless of whether oxygen or sulfur are coordinated at the β position, for MgATPβS we have

$$K_{obsd} = (K_{\beta os} + K_{\beta so})(1 + K_{\alpha oo}) \quad (12)$$

where $K_{\beta os}$ and $K_{\beta so}$ are stability constants for formation of bidentate chelates with oxygen (with sulfur being the noncoordinated atom) or with sulfur (with oxygen being the noncoordinated atom) and $K_{\alpha oo}$ has the same meaning as in eq 11. Since $K_{\beta so}$ is very small for Mg^{2+}, the ratio of stability constants for MgATP and MgATPβS (4.6) gives 2.3 as the ratio of $K_{\beta oo}$ and $K_{\beta os}$. In view of the standard errors of the stability constants used in the calculations, this value is probably not significantly different from that calculated above for MgADPαS.

Equations 11 and 12 will hold for CdATP and CdATPβS, except that $K_{\beta oo}$, $K_{\beta os}$, $K_{\beta so}$, and $K_{\alpha oo}$ will have values characteristic for Cd^{2+}, as opposed to Mg^{2+}. The ratio between the stability constants will now be $2K_{\beta oo}/(K_{\beta os} + K_{\beta so})$, and if we assume the ratio $K_{\beta oo}/K_{\beta os}$ to be 2.3 by analogy with the MgATP and MgATPβS values, the ratio of $K_{\beta so}$ and $K_{\beta os}$, which is the ratio of Cd–S to Cd–O coordination, is 54.

When the errors in the logs of the stability constants are taken into account in these calculations, the errors in the ratios of Cd–S to Cd–O coordination might be as high as a factor of 2. We will thus average the two values obtained for CdADPαS and CdATPβS and adopt a value of 60 for this ratio. Despite the considerable uncertainty about the exact value of this ratio, this is the first attempt to estimate it quantitatively, and we believe it can be used with the understanding that the value is known only to the first significant figure.

Table IV: Ratio of Oxygen to Sulfur Coordination for Metal Ions in Complexes with ATPβS[a]

metal ion	oxygen/sulfur coordination: from eq 16 and 17	oxygen/sulfur coordination: from V/K ratios[b]
Mg^{2+}	31000	31000
Ca^{2+}	3100	3900
Mn^{2+}	158	193
Cd^{2+}	0.018[c]	

[a] Calculated from the data of Jaffe & Cohn (1979) by the methods given in the text, except for the value for Cd^{2+}. [b] [Ratio of V/K values for isomers B and A of ATPβS] × 11.37 (see text for rationale). [c] From the present work.

For ATPαS the analysis is more complex. The stability constants for MgATP and MgATPαS differ only by a factor of 1.7, which is possibly not significant. The value for MgATP is given by eq 11, while that for MgATPαS is given by

$$K_{obsd} = K_{\beta oo}(2 + K_{\alpha os} + K_{\alpha so}) \quad (13)$$

Because of the very small contribution from sulfur coordination at the α position, we can set $K_{\alpha so}$ equal to zero in this equation. Then if we assume that $K_{\alpha os}$ is lower than $K_{\alpha oo}$ by a factor similar to that seen for MgADPαS, or for $K_{\beta os}$ vs. $K_{\beta oo}$ for MgATPβS, we can replace $K_{\alpha os}$ by $K_{\alpha oo}$ divided by 1.8 (the average of the values for MgADPαS and MgATPβS) to give

$$1.7 = \frac{2K_{\beta oo}(1 + K_{\alpha oo})}{K_{\beta oo}(2 + K_{\alpha oo}/1.8)} \quad (14)$$

which yields $K_{\alpha oo} = 1.33$ and $K_{\alpha os} = 0.74$. These values correspond to 57% and 27% tridentate coordination in MgATP and MgATPαS. The degree of tridentate coordination in MgATP has been estimated by Huang & Tsai (1982) as ~50% on the basis of ^{17}O NMR studies of the line broadening caused by Mg^{2+} coordination, and thus, despite the large error involved in these calculations, they seem to give the correct order of magnitude estimate for the tridentate content of these complexes.

A more precise calculation can be made of the degree of tridentate coordination in CdATP and CdATPαS. The stability constants for these complexes differ by a factor of 3.63. If we assume that $K_{\alpha oo}$ is greater than $K_{\alpha os}$ by a factor of 1.8 as we did with the Mg^{2+} complexes and further assume that $K_{\alpha so}$ is 60 times $K_{\alpha os}$ (by analogy with our values for CdADPαS and CdATPβS), then the ratio of eq 13 and 11 gives

$$3.63 = \frac{K_{\beta oo}(2 + 61K_{\alpha oo}/1.8)}{2K_{\beta oo}(1 + K_{\alpha oo})} \quad (15)$$

from which $K_{\alpha oo} = 0.20$, $K_{\alpha os} = 0.10$, and $K_{\alpha so} = 6.6$. These values give 16% tridentate for CdATP and 76% tridentate (sulfur coordinated at α), 1.3% tridentate (oxygen coordinated at α), and 23% bidentate for CdATPαS. The lower tridentate character of CdATP than of MgATP may reflect the competition from coordination to N-7 in the former, but not the latter.

Estimation of Oxygen/Sulfur Coordination Ratios for ATPβS with Other Metal Ions. The work of Jaffe & Cohn (1979) allows one to estimate the degree of oxygen vs. sulfur coordination of ATPβS with metal ions other than Cd^{2+} as follows. If one compares the kinetic constants of ATP and ATPβS (isomer B; the one that gives the active Λ screw sense

isomer with Mg^{2+}) as substrates for yeast hexokinase with Mg^{2+}, one sees that substitution of sulfur for oxygen in the noncoordinated β position lowers V_{max} by a factor of 27.7 and raises the Michaelis constant by a factor of 2.7.[7] The effect of sulfur in the coordinated β position comes from comparison of the kinetics with Cd^{2+} of ATP and ATPβS (isomer A; the one that gives the Λ isomer when the sulfur is coordinated); V_{max} drops by a factor of 5.09, and the Michaelis constant rises by a factor of 1.29. Let us now consider the activity of isomer B of ATPβS with Cd^{2+}. We know from our work that only one part in 60 will be the active Λ screw sense isomer, and thus, if only this isomer bound to the enzyme, the Michaelis constant would be elevated by a factor of 60, as well as by the expected factor of 2.7 for the noncoordinated sulfur at the β position. The actual Michaelis constant is lower than the one calculated in this way by a factor of 16, showing that the inactive Δ screw sense isomer binds nonproductively to the enzyme [this is not surprising, since the same thing was observed with CrATP isomers by Dunaway-Mariano & Cleland (1980a)]. Since nonproductive binding by a portion of the substrate lowers both V_{max} and the Michaelis constant by the same factor, we expect V_{max} in this case to be decreased by this factor, as well as by the factor of 27.7 for noncoordinated sulfur at the β position. The expected V_{max} is then 0.013 μmol min^{-1} mg^{-1}, compared to an observed value of 0.03. The agreement is excellent, especially since any cross-contamination of isomer A in ATPβS would raise the observed V_{max} value.

The success of this analysis allows us to consider the V_{max} values observed for the A isomer of ATPβS with various metal ions. The factor by which V_{max} and the Michaelis constant will be reduced by nonproductive binding of the predominant (with Mg^{2+}) Δ screw sense isomer is

$$\text{NPBF} = 1 + (K_\Lambda/[\Lambda])/(K_\Delta/[\Delta]) \quad (16)$$

where $[\Lambda]$ and $[\Delta]$ are the concentrations of the active Λ and inactive Δ screw sense isomers and K_Λ and K_Δ are the Michaelis constant and dissociation constant for these isomers. We want to solve for the $[\Delta]/[\Lambda]$ ratio, which is the oxygen/sulfur coordination ratio. When oxygen coordination dominates, K_Δ is simply the Michaelis constant for the A isomer, while K_Λ is the Michaelis constant for ATP multiplied by 1.29 to correct for the effect of coordinated sulfur. The relationship between the observed V_{max} for the A isomer and that with ATP is then

$$V_{max}(\text{obsd}) = (V_{max} \text{ for ATP})/(5.09\text{NPBF}) \quad (17)$$

where the factor 5.09 corrects for the effect of coordinated sulfur. When eq 16 and 17 are solved for the oxygen/sulfur ratio, the values in Table IV are obtained. The estimate of 31 000 for Mg^{2+} is a minimal value, since any cross-contamination of the B isomer in the A one would give too small a value.

Another way to approach the estimation of oxygen/sulfur coordination ratios for metals other than Mg^{2+} or Cd^{2+} is to compare the V/K ratios for isomers A and B of ATPβS with different metals. We have concluded above that the V/K ratio for isomer A will be reduced by the coordinated sulfur by factors of 5.09 (on V_{max}) and 1.29 (on the Michaelis constant) for an overall effect of 6.57, while V/K for the B isomer is reduced by the noncoordinated sulfur by factors of 27.7 and 2.7 for an overall effect of 74.7 The observed ratio of V/K values for isomers B and A must thus be multiplied by 74.7/6.57 = 11.37 to correct for the differential effects of sulfur in coordinated and noncoordinated positions. Values calculated in this way are also listed in Table IV. The value for Mg^{2+} is of course the same as that estimated by the previous method, since the same kinetic constants are used in both analyses, but the estimates for Ca^{2+} and Mn^{2+} involve different kinetic constants, and the agreement is gratifying. This analysis cannot be validly applied to the kinetic data for Co^{2+}, Ni^{2+}, and Zn^{2+}, since product release appeared to be partly rate limiting in these cases and the Michaelis constants for ATPβS isomers were lower than expected on the basis of the values for ATP. The values from the relative V/K analysis do suggest oxygen/sulfur coordination ratios of 5–9, however.

Comments on Mg^{2+}–Cd^{2+} Reversal Studies with Thionucleotides. The above calculations show the importance when determining Mg^{2+}–Cd^{2+} reversals of specificity with sulfur-substituted nucleotides of doing a full kinetic analysis, and the oxygen/sulfur coordination ratios from the present work should permit such determinations to be placed on a sound footing. One must be careful, however, *not* to depend solely on V_{max} ratios for determining screw sense specificity. The correct prediction of specificity by Jaffe & Cohn (1979) from V_{max} values resulted from the extensive nonproductive binding of the inactive Δ isomers to hexokinase. In the absence of nonproductive binding of the inactive isomers, V_{max} with Cd^{2+} for the less active of the enantiomers of the thionucleotide would be determined by the small level of active screw sense isomer coming from Cd–O coordination, or from contamination with the opposite diastereomer. If, as was the case with hexokinase, sulfur substitution in the noncoordinated position caused a greater drop in V_{max} than in the coordinated position, one would not see a reversal of isomer specificity with Cd^{2+} based on V_{max} values (the B isomer would be 5-fold slower than the A isomer with either metal ion), although the K_m would be greatly elevated for the less active diastereomer (isomer A with Mg^{2+} and isomer *b* with Cd^{2+}).

The correct kinetic parameter to compare is V/K, and it is necessary to determine V/K values for each metal with ATP as well as with both isomers of thionucleotide. Analysis as we have done above will then yield (a) the screw sense specificity of the enzyme and (b) the effect on V_{max} and K_m of sulfur substitution in coordinated and noncoordinated positions. The oxygen/sulfur coordination ratios for Mg^{2+}, Ca^{2+}, and Mn^{2+} that we have derived for ATPβS can be used with some confidence, but a similar study with ADPαS isomers and creatine kinase needs to be carried out to determine oxygen/sulfur coordination ratios for ADPαS with Mg^{2+}, Ca^{2+}, and Mn^{2+}. The use of ATPαS poses special problems, since our

[7] These factors are fairly constant for different metal ions (Jaffe & Cohn, 1979). Thus, V_{max} for ATPβS (isomer B) is decreased below that for ATP by factors of 25 with Ca^{2+} and 33 with Mn^{2+}, both of which coordinate to oxygen in preference to sulfur. The factors with Ni^{2+}, Zn^{2+}, and Co^{2+} range from 24 to 52. The Michaelis constant for ATPβS (isomer B) is higher than that for ATP by factors of 2.4 with Ca^{2+} and 1.8 with Mn^{2+}. The ratios with the other three ions run from 0.8 to 10 and are probably affected by rates of product release. These calculations ignore the fact that MgATP and CdATP will consist of nearly equal amounts of Λ and Δ isomers, while the ATPβS complexes are predominantly one screw sense or the other. If the Δ isomers of MgATP and CdATP bind nonproductively to hexokinase, the observed V will be lower than that for the fully active Λ isomers, while if nonproductive binding is too weak to be important, V is unaffected, but the true K_m of the Λ isomer is half of the observed value. As long as the proportion of Λ and Δ isomers and the degree of nonproductive binding are the same for MgATP and CdATP, all of these corrections cancel out and one obtains the same answers we give in the text and Table IV. Scheller et al. (1981) report that 54% of CdATP has N-7 of adenine coordinated to the metal, and if the ability to form this additional bond differed for the Λ and Δ isomers, an error would be introduced. Examination of models shows that both isomers can form N-7 chelates, so in the absence of experimental evidence to the contrary, we will assume equal coordination to N-7 in both isomers.

Table V: Line Widths and Exchange Rate Constants for Mg^{2+} with ADP and ATP[a]

complex	pH	[MgAXP][b] (mM)	[dimer][c] (mM)	line width[d] (Hz)	$1/\tau$ (s^{-1})
MgADP	5.71	1.91	0.02	31.3	9700
		9.13	0.31	19.5	16680
		14.3	0.86	14.8	23200
		19.3	1.48	9.9	39680
		22.7	1.93	7.5	60240
				11.4	32700[e]
				5.2	120500[f]
		24.3	2.33	7.7	57800
MgATP	5.95	1.88	0.008	43.7	9800
		9.81	0.14	38.1	11360
		14.9	0.40	31.8	13680
		22.8	0.86	24.9	17800
				39.3	10990[g]
				13.3	35710[f]
		32.3	1.59	16.1	28570
		39.8	2.05	13.4	35340

[a] 32 °C, 0.1 M KNO_3. [b] The HAXP concentration was equal to that of MgAXP is all cases. [c] It was assumed that the [dimer]/([HAXP][MgAXP]) ratio is the average of the HAXP and MgAXP dimer stability constants reported by Scheller et al. (1981) and Scheller & Sigel (1983). [d] The line widths of HADP and HATP at pH 4.5 and the absence of exchange were 1.70 and 1.20 Hz, respectively. MgADP and MgATP complexes at pH 7.0 had natural line widths of 4.21 and 4.50 Hz, respectively. For the broadened resonances the Nicolet NMCCAP curve analysis/deconvolution program allowed determination of the line widths of the downfield peak of the doublets corresponding to the β- and γ-phosphates of ADP and ATP, respectively. [e] 13 °C. [f] 40 °C. [g] 12 °C.

data suggest that CdATPαS exists as ~75% tridentate species (almost all with Cd–S coordination), compared with ~16% tridentate coordination with CdATP. In contrast, MgATPαS is probably ~27% tridentate, while MgATP may be 50–57% tridentate on the basis of our data and that of Huang & Tsai (1982). Therefore, the ability of the MgATPαS and CdATPαS complexes to act as alternate substrates will be dependent not only on screw sense specificity but also on the ability of an enzyme to accept tridentate vs. bidentate complexes and its preference for the compressed vs. extended form of the nucleotide at the active site.

Association and Dissociation Rate Constants for Mg^{2+} with ATP and ADP. A line broadening which could not be attributed to paramagnetic ions was observed for the terminal phosphorus peaks of ATP and ADP in the ^{31}P NMR spectrum (Figure 7). Conditions of pH and Mg^{2+} concentration that produced nucleotides either fully protonated or fully complexed to Mg^{2+} gave no broadening. Further confirmation that the observed broadening is due to dynamic exchange is demonstrated by the temperature dependence of the NMR line width (Table V). Only when both HAXP and MgAXP (X is D for ADP and T for ATP) were present in solution was the broadening apparent. These observations are readily interpreted in terms of dynamic exchange (Becker, 1983; Jackman & Cotton, 1975). According to the theoretical treatments of exchange as they relate to NMR, "slow" exchange would give narrow and separate peaks for HAXP and MgAXP, "fast" exchange would yield a single narrow peak of intermediate chemical shift, but "intermediate" exchange would give a broadened peak of intermediate chemical shift, the width of which depends on both the rate of exchange and the separation of the parent peaks. Various derivations for the quantitative relationship between this broadened line width, the frequency difference of the peaks in the absence of exchange, the relative population of the two species, the natural line widths of the two species, and the exchange rate ($1/\tau$) are given by Jackman & Cotton (1975). We have chosen to use the Nicolet two-site

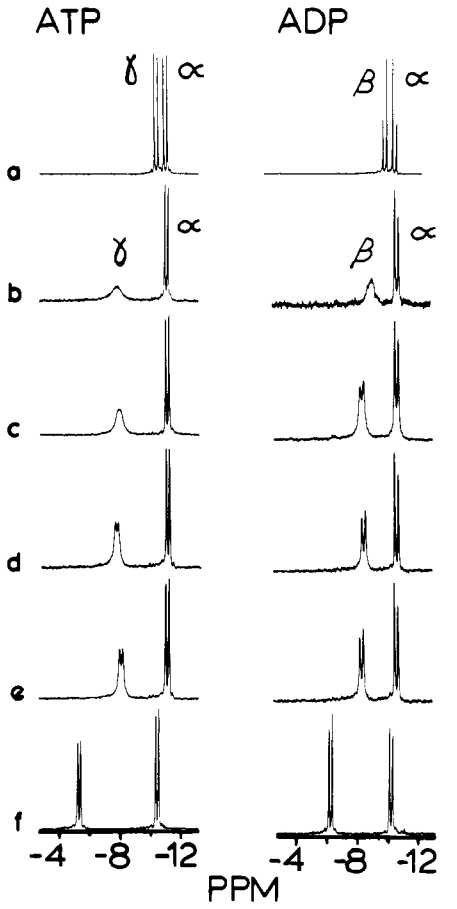


FIGURE 7: Concentration dependences of ^{31}P NMR line widths for MgATP (left) and MgADP (right). For MgATP (only α and γ resonances are shown): spectrum a [ATP] = 55 mM, pH 4.5, no Mg^{2+}; spectra b–e, pH 5.95, 32 °C, μ = 0.1 M, [Mg^{2+}] = 0.5[ATP], [ATP] = (b) 3.8 mM, (c) 27.1 mM, (d) 54.7 mM, (e) 63.2 mM; spectrum f, [ATP] = 55 mM, pH 8, [Mg^{2+}] = 80 mM. For MgADP: spectrum a, [ADP] = 55 mM, pH 4.5, no Mg^{2+}; spectra b–e, pH 5.71, 32 °C, μ = 0.1 M, [Mg^{2+}] = 0.5[ADP], [ADP] = (b) 5.0 mM, (c) 18.0 mM, (d) 40.0 mM, (e) 50.0 mM; spectrum f, [ADP] = 55 mM, pH 7, [Mg^{2+}] = 80 mM.

exchange simulation routine (NMCXCH program of the 1280 Nicolet Data Acquisition System) to establish a simple relationship between the exchange rate and experimental line widths rather than using the more rigorous density matrix formalism. Because the degree of spin coupling between the two species is invariant and since the P–O–P bonds are never broken, the error introduced by neglecting the contribution of mixed wave functions for spin coupling is estimated to be less than the systematic errors of the experiment. Experimental line widths were determined by using the Nicolet deconvolution program NMCCAP. When the exchange-broadened line width was greater than the phosphorus coupling constant (J = 17 Hz), simulated spectra consisting of two peaks with equal areas and a fixed separation of 17 Hz were generated. The goodness of fit to the observed experimental line shape was evaluated by least-squares methods. The parameters required for this simulation[8] are line positions and widths in

[8] The equation used to calculate the exchange simulation is

$$y_x = K[P(1 + \tau C) + QR]/(P^2 + R^2)$$

where $P = \tau(J - E^2 + B^2) + G$, $Q = \tau(E - D)$, and $R = E[1 + \tau(W_1 + W_2)] + D + \tau B(W_2 - W_1)$. $A = \pi(F_1 + F_2)$, $B = \pi(F_1 - F_2)$, $C = N_1W_2 + N_2W_1$, $G = N_1W_1 + N_2W_2$, $D = B(N_1 - N_2)$, $E = A - x$, $x = 2$ (frequency in Hz), F_1 = frequency A, F_2 = frequency B, $J = W_1W_2$, K = scaling factor, N_1 = fraction A, $N_2 = 1 - N_1$, $\tau = 1/$(exchange rate), $W_1 = \pi$(line width of A), and $W_2 = \pi$(line width of B).

Table VI: Association and Dissociation Rate Constants for Mg^{2+} with ADP and ATP[a]

rate constant	nucleotide	
	ADP	ATP
k_1 (M^{-1} s^{-1})	$8.6 \pm 3.3 \times 10^7$	$3.5 \pm 0.3 \times 10^8$
k_2 (s^{-1})	$6.7 \pm 2.6 \times 10^3$	$6.9 \pm 0.6 \times 10^3$
k_3 (M^{-1} s^{-1})	$2.3 \pm 0.9 \times 10^9$	$3.5 \pm 0.3 \times 10^9$
k_4 (M^{-1} s^{-1})	$6.4 \pm 2.8 \times 10^6$	$4.2 \pm 0.3 \times 10^7$
k_5 (s^{-1})	1.1×10^4	1.2×10^4
k_6 (M^{-1} s^{-1})	5.0×10^{10}	5.0×10^{10}
k_7 (M^{-1} s^{-1})	$2.3 \pm 0.3 \times 10^7$	$1.24 \pm 0.06 \times 10^7$

[a] 32 °C, μ = 0.1 M KNO_3.

the absence of exchange (readily determined from samples at low pH or high Mg^{2+} concentration), the relative populations of the two species HAXP and MgAXP (determined from the thermodynamic constants reported in this work), and the mean lifetime of the phosphorus nucleus in each species, τ (the reciprocal of the exchange rate). When τ was varied and the line width of the peak in the simulated spectrum was determined, the following relationship was established for the MgATP–HATP exchange:

$$\tau = [0.2442(\text{line width}) - 0.42]/10^5 \quad (18)$$

and for the MgADP–HADP exchange:

$$\tau = [0.3625(\text{line width}) - 1.06]/10^5 \quad (19)$$

τ is in seconds and line width in hertz.

In terms of the individual rate constants associated with the exchange

$$H^+ + MgAXP \underset{k_4}{\overset{k_3}{\rightleftharpoons}} Mg^{2+} + HAXP \quad (20)$$

the reciprocal of τ is equal to the sum of the pseudo-first-order rate constants:

$$1/\tau = k_3[H^+] + k_4[Mg^{2+}_{free}] \quad (21)$$

By use of the stability constant (K_{ML}) and the pK_a for protonation of the nucleotide, the equilibrium constant for eq 20 can be expressed as

$$K_{eq} = 1/(K_{ML}K_a) \quad (22)$$

and therefore

$$k_4 = (1/\tau)[1/(K_{eq}[H^+] + [Mg^{2+}_{free}])] \quad (23)$$

$$k_3 = k_4K_{eq} \quad (24)$$

When the following more detailed scheme for the exchange is considered

$$HAXP \underset{k_6[H^+]}{\overset{k_5}{\rightleftharpoons}} AXP \underset{k_2}{\overset{k_1[Mg]}{\rightleftharpoons}} MgAXP \quad (25)$$

the association rate constant k_1 and the dissociation constant k_2 are given by

$$k_1 = k_4k_6[H^+]/(k_5 - k_4[Mg]) \quad (26)$$

$$k_2 = k_1/K_{ML} \quad (27)$$

where k_6 is the diffusion-limiting bimolecular rate constant for proton transfer and $k_5 = k_6K_a$. These rate constants for ADP and ATP are in Table VI.

A dependence of $1/\tau$ on the concentration of nucleotide is observed which is not predicted by eq 21. When nucleotide and Mg^{2+} are increased simultaneously so that the ratio of HAXP/MgAXP remains close to unity (the pH and level of free Mg^{2+} remain constant), the value of $1/\tau$ increases as

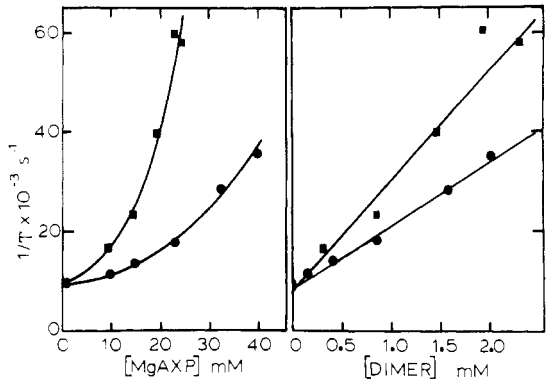


FIGURE 8: Dependence of $1/\tau$ on [HAXP] (left panel) and [dimer] (right panel). (●) ATP; (■) ADP. Dimer concentrations were calculated by using stability constants from Scheller et al. (1981) and Scheller & Sigel (1983a,b).

shown in Figure 8 (left panel). Scheller et al. (1981) and Scheller & Sigel (1983b) report the existence of dimers in concentrated nucleotide solutions and give equilibrium constants for dimerization of ADP and ATP. The parabolic curves of Figure 8 (left panel) become linear when $1/\tau$ is plotted vs. dimer concentration (Figure 8, right panel). It appears that the exchange rate of Mg^{2+} is increased either by trimer formation or by direct bimolecular reaction of a dimer and a monomeric nucleotide, and a more complete expression for the overall exchange rate is

$$1/\tau = k_3[H^+] + k_4[Mg^{2+}_{free}] + k_7[\text{dimer}] \quad (28)$$

The limiting value for the exchange in dilute solutions of nucleotide is obtained from the vertical intercept of the right panel Figure 8. This value was used to calculate k_3 and k_4, and k_7 was determined from the slope (see Table VI). The dissociation rate constants for MgADP and MgATP are both equal to 7×10^3 s^{-1} at 32 °C. The bimolecular association rate constants are $(8.6 \pm 3.3) \times 10^7$ M^{-1} s^{-1} and $(3.5 \pm 0.3) \times 10^8$ M^{-1} s^{-1} for ADP + Mg^{2+} and ATP + Mg^{2+}, respectively. Since these rate constants are far higher than the water exchange rate of $\sim 10^5$ s^{-1} for Mg^{2+} (Geier, 1963), the formation of MgADP and MgATP proceeds via an associative, rather than a dissociative, process (Basolo & Pearson, 1967).

For the above calculations a value of 5×10^{10} M^{-1} s^{-1} was assumed for k_6 (Jencks, 1969). Equation 26 places a lower limit of 2.2×10^{10} M^{-1} s^{-1} on this rate constant. For values of k_6 less than 4×10^{10} but greater than 2.2×10^{10} M^{-1} s^{-1}, k_2 is quite sensitive to the assumed value of k_6 (k_2 varies from 8×10^3 to 8×10^4 s^{-1} over this range of k_6 values), but for the range of k_6 values from 5×10^{10} to 10×10^{10} M^{-1} s^{-1}, k_2 only varies from 7×10^3 to 5×10^3 s^{-1}.

Two previous reports of the dissociation rate constants for MgATP have appeared. Values for k_1 of 1.5×10^7 M^{-1} s^{-1} and for k_2 of 1500 s^{-1} were reported by Diebler et al. (1960) by using T-jump experiments and low enough levels of MgATP that no dimer was present. The fact that the MgATP stability constant used was 10^4 explains why k_1 was an order of magnitude lower than the value reported here, while the use of 20 °C rather than 32 °C explains why k_2 was lower than our value of 6900 s^{-1}. Bryant (1972) used ^{25}Mg NMR to determine the mean residence time of Mg^{2+} in ATP but did not calculate k_1 or k_2. Under his conditions ($[Mg^{2+}]$ = 1.5 M, $[ATP]_t$ = 19 mM, pH 6) he reported $1/\tau$ = 20 000 s^{-1}, which in this case should be given by an equation of the form

$$1/\tau = k_1[ATP]_{free} + k_2 + k_7'[\text{dimer}] \quad (29)$$

where k_1 and k_2 are the same rate constants shown in eq 25

but k_7' may not equal k_7 in eq 28, since the exchange processes are different in the two experiments. If we use our values for k_1 and k_2 and the concentrations of Bryant (1972), the first two terms in eq 29 have values of 88 and 6900 s^{-1}, and thus, k_7'[dimer] would have to be about 13 000 s^{-1}. With our value of k_7, k_7[dimer] would be 21 600 s^{-1}, so k_7' appears to be 60% of k_7. Unfortunately we cannot confirm these calculations, since Bryant (1972) did not measure τ as a function of nucleotide concentration, but it seems certain that exchange involving the dimer was a major contribution to his $1/\tau$ value.

Acknowledgments

We thank Drs. Jean van Pelt and Perry Frey for supplying quantities of ATPαS and ADPαS.

Registry No. ATP, 56-65-5; ADP, 58-64-0; (*S*)-ATPαS, 58976-48-0; (*R*)-ATPβS, 59261-35-7; ATPγS, 35094-46-3; (*S*)-ADPαS, 59286-20-3; ADPβS, 35094-45-2; MgATP, 1476-84-2; MgADP, 7384-99-8; (*S*)-MgATPαS, 72052-16-5; (*R*)-MgATPβS, 72052-08-5; (*S*)-MgADPαS, 79189-53-0; CdATP, 72052-13-2; CdADP, 79189-47-2; (*S*)-CdATPαS, 72052-18-7; (*R*)-CdATPβS, 72052-15-4; (*S*)-CdADPαS, 79189-48-3.

References

Adolfsen, R., & Moudrianakis, E. N. (1978) *J. Biol. Chem. 253*, 4378.

Armstrong, V. W., Yee, D., & Eckstein, F. (1979) *Biochemistry 18*, 420.

Basolo, F., & Pearson, R. G. (1967) *Mechanisms of Inorganic Reactions*, Wiley, New York.

Becker, E. D. (1980) *High Resolution NMR: Theory and Chemical Applications*, Academic Press, New York.

Bryant, R. G. (1972) *J. Magn. Reson. 6*, 159.

Cohn, M. (1982) *Acc. Chem. Res. 15*, 326.

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

Cornelius, R. D., Hart, P. A., & Cleland, W. W. (1977) *Inorg. Chem. 16*, 2799.

Cotton, F. A., & Wilkenson, G. (1972) *Advanced Inorganic Chemistry*, 3rd ed., Interscience, New York.

Diebler, H., Eigen, M., & Hammes, G. G. (1960) *Z. Naturforsch. B: Anorg. Chem., Org. Chem., Biochem., Biophys., Biol. 15B*, 554.

Dunaway-Mariano, D., & Cleland, W. W. (1980a) *Biochemistry 19*, 1496.

Dunaway-Mariano, D., & Cleland, W. W. (1980b) *Biochemistry 19*, 1506.

Eckstein, F. (1975) *Angew. Chem., Int. Ed. Engl. 14*, 160.

Eckstein, J., & Goody, R. S. (1976) *Biochemistry 15*, 1685.

Frey, C. M., & Stuehr, J. E. (1972) *J. Am. Chem. Soc. 94*, 8898.

Geier, G. (1963) *Ber. Bunsenges. Phys. Chem. 69*, 617.

Gerlt, J. A., Reynolds, M. A., Demou, P. C., & Kenyon, G. L. (1983) *J. Am. Chem. Soc. 105*, 6469.

Huang, S. L., & Tsai, M.-D. (1982) *Biochemistry 21*, 951.

Jackman, L. M., & Cotton, F. A., Eds. (1975) *Dynamic NMR Spectroscopy*, Academic Press, New York.

Jaffe, E. K., & Cohn, M. (1978a) *Biochemistry 17*, 652.

Jaffe, E. K., & Cohn, M. (1978b) *J. Biol. Chem. 253*, 4823.

Jaffe, E. K., & Cohn, M. (1979) *J. Biol. Chem. 254*, 10839.

Kahn, M. M. T., & Martell, A. E. (1967) *J. Am. Chem. Soc. 89*, 5586.

Martell, A. E., & Schwarzenbach, G. (1956) *Helv. Chim. Acta 39*, 653.

Martell, A. E., & Smith, R. H. (1976) in *Critical Stability Constants*, Plenum Press, New York.

Merritt, E. A., Sundaralingam, M., Cornelius, R. D., & Cleland, W. W. (1978) *Biochemistry 17*, 3274.

O'Sullivan, W. J., & Perrin, D. D. (1964) *Biochemistry 3*, 18.

Phillips, R. (1966) *Chem. Rev. 66*, 501.

Raushel, F. M., Anderson, P. M., & Villafranca, J. J. (1978) *J. Biol. Chem. 253*, 6627.

Rossotti, F. J. C., & Rossotti, H. (1961) *Determination of Stability Constants, and Other Equilibrium Constants in Solution*, McGraw-Hill, New York.

Scheller, K. H., & Sigel, H. (1983a) *Inorg. Chim. Acta 79*, 242.

Scheller, K. H., & Sigel, H. (1983b) *J. Am. Chem. Soc. 105*, 5891.

Scheller, K. H., Hofstetter, F., Mitchell, P. R., Prits, B., & Sigel, H. (1981) *J. Am. Chem. Soc. 103*, 247.

Schwarzenbach, G., & Schwarzenbach, K. (1963) *Helv. Chim. Acta 46*, 1390.

Sheu, K.-F. R., & Frey, P. A. (1977) *J. Biol. Chem. 252*, 4445.

Smith, R. M., & Alberty, R. A. (1956) *J. Am. Chem. Soc. 78*, 2376.

Welcher, T. J. (1958) *The Analytical Uses of Ethylenediamine Tetraacetic Acid*, Van Nostrand, Princeton, NJ.