Interactions of Metal Ions with μ-Monothiopyrophosphate†

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Received June 10, 1991; Revised Manuscript Received July 30, 1991

ABSTRACT: μ-Monothiopyrophosphate (MTP) binds monovalent and divalent metal ions with dissociation constants (K_d) similar to those for pyrophosphate (PP_i). The values of K_d for metal–MTP complexes are the following, as measured kinetically in the hydrolysis of MTP (μM): Mg^{2+}, 32 ± 4; Mn^{2+}, 5.4 ± 1.4; and Co^{2+}, 27 ± 15. The thermodynamically measured (EPR) values for Mg^{2+} and Co^{2+} are 28 ± 13 μm and 11 ± 4 μM, respectively; and the K_d for the complex $MnPP_i$ is 3.4 ± 0.5 μM. The metal–MTP complexes undergo hydrolysis at rates modestly faster or slower than the rate at which MTP itself reacts. The complexes $MgMTP^{2-}$, $CoMTP^{2-}$, and $MnMTP^{2-}$ undergo hydrolytic cleavage with release of thiophosphate with observed first-order rate constants of 1.6×10^{-2} min^{-1}, 2.3×10^{-2} min^{-1}, and 0.6×10^{-2} min^{-1}, respectively, at 35 °C, compared with 1.1×10^{-2} min^{-1} for MTP^{4-} under the same conditions. Alkali metal cations also stimulate or retard the hydrolysis of MTP. At 25 °C and pH 12.2, the observed rate constant for tetramethylammonium MTP^{4-} is 2.1×10^{-3} min^{-1}, and the estimated rate constants (min^{-1}) for saturating alkali metals under the same conditions are as follows: Li^+, 0.25×10^{-3}; Na^+, 3.9×10^{-3}, K^+, 6.7×10^{-3}; and Cs^+, 6.7×10^{-3}. Divalent metal ions markedly retard the hydrolysis of MTP at pH 7 and 8 because complexation shifts the pH rate profile more than 2 pH units toward the acid side. Alkali metal ions have similar effects on the hydrolysis of MTP in neutral and alkaline solutions. The entropies of activation (ΔS^*) for MTP^{4-}, $HMTP^{3-}$, and $MgMTP^{2-}$ are +14.1, +0.2, and −12.3 cal·deg^{-1}·mol^{-1}, respectively. These values are consistent with monomolecular processes, with the differences most likely resulting from differences in solvation of ground and transition states. The enthalpies of activation (ΔH^*) for this series are 27.6, 19.7, ad 19.3 kcal·mol^{-1}, respectively. The modest effects of metal ions on the hydrolysis of MTP do not suggest that metal complexation perturbs the mechanism of hydrolytic P–S bond cleavage in an important way.

μ-Monothiopyrophosphate (MTP)[1] is an analogue of PP_i in which the bridging oxygen is replaced by sulfur (Loewus & Eckstein, 1983). The geometric and chemical differences between MTP and PP_i make MTP a potentially useful analogue for mechanistic studies of the many enzymes that utilize PP_i as a substrate or produce it as a product. Nearly all of these enzymes utilize or produce $MgPP_i$ rather than PP_i itself; therefore, in order to compare the interactions of MTP and PP_i with enzymes, knowledge of how MTP interacts with metal ions is required. We also need to know the effects of metal ions on the chemical properties of MTP. We here describe the interactions of MTP and PP_i with monovalent and divalent metal ions. The dissociation constant for complexes of various metal ions with MTP and PP_i are reported, as well as the effects of monovalent and divalent cations on the rate at which MTP undergoes hydrolysis.

Experimental Procedures

Materials. Divalent metal chlorides were the highest grade available from Aldrich, greater than 99.99% pure. Metal concentrations for binding studies were determined by EDTA titrations using the following dyes: Calmagite in NH_4Cl/NH_4OH at pH 10 (Mg^{2+}), Methyl Thymol Blue in hexamethylenetetramine at pH 6.5 (Co^{2+}), and Arsenazo I in pyridine hydrochloride at pH 8.0 (Mn^{2+}). The EDTA solutions were titrated with atomic absorption standards (Aldrich); their concentrations were found to be constant regardless of which assay was used.

Tetramethylammonium chloride (Aldrich) was washed with $CHCl_3$, recrystallized from alcohol, and dried in vacuo at 55 °C for at least 8 h. All hydroxide solutions were standardized by titration against potassium hydrogen phthalate, which had been dried overnight at 120 °C. $(CH_3)_4NOH$ was a 10% (w/v) solution from Aldrich and was filtered through a coarse sintered glass funnel before use. Lithium hydroxide was synthesized under argon from lithium metal (Aldrich) and water and was filtered through sintered glass. HCl was distilled, and the constant-boiling fraction was taken. LiCl concentrations for the NMR titrations were determined using the Thorin I lithium assay (Thomason, 1956). The standard curve was determined using an atomic absorption standard (Aldrich).

EPR experiments were done on a Varian E-3 X-band instrument. NMR experiments were done on a Bruker AM500 spectrometer with either a 5- or a 10-mm broad-band probe. Kinetic measurements of MTP hydrolysis were made spectrophotometrically at 226–228 nm using either of the following instruments: a Varian Cary 118C recording spectrophotometer equipped with a Forma Model 2160 circulating bath and jacketed cells, or a HP 8452A diode array spectrophotometer equipped with a Hewlett Packard HP 89500A Chemstation, a Lauda K-2/R circulating bath, and a HP 89075C Multicell Transport.

Sample Preparation and Analysis. Li_4MTP was synthesized by a modification of the procedure of Loewus and Eckstein (Halkides, et al., 1991); Li_4PPi was prepared from Na_4PP_i by ion exchange by the same procedure as that used for the last step in the preparation of Li_4MTP. The tetra-

† This work was supported by Grant GM30480 from the National Institute of General Medical Sciences.

[1] Abbreviations: MTP, μ-monothiopyrophosphate; PP_i, pyrophosphate; MOPS, 3-(*N*-morpholino)propanesulfonate; EPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonate.

0006-2960/91/0430-10307$02.50/0

methylammonium salts were prepared by adsorbing Li_4MTP to a small column of Dowex AG1-X8 in the chloride form and eluting with a solution of 0.1 M $(CH_3)_4NCl$ and 0.01 M $(CH_3)_4NOH$. The lithium contamination in $[(CH_3)_4N]_4MTP$ was found by ^7Li NMR to be about 0.03 mol of Li/mol of MTP.

Methylenediphosphonic acid was dissolved with $(CH_3)_4NOH$ to pH 8. This solution was extracted first with diphenylthiocarbazone in $CHCl_3$ to remove contaminating metals and then with $CHCl_3$. The solution was brought to pH 13.5 with additional $(CH_3)_4NOH$.

Water, KCl, and the NMR sample from the KCl titration of MTP were evaluated for contaminating metals by plasma emission analysis, which showed that Ca, Mg, Zn, Mn, Fe, Cu, Al, Na, Cd, Cr, Mo, Ni, Co, As, Pb, and Se were undetectable. Trace amounts of B were present in all three samples, and P, S, and K were present in the expected amounts.

Kinetic Methods. The hydrolysis of MTP was measured spectrophotometrically by monitoring the formation of thiophosphate at wavelengths between 232 and 215 nm. The pH was measured at the temperature of the experiment. When the HP spectrophotometer system was used, the initial velocities and first-order rate constants were calculated by use of the Pascal-based software in the Chemstation. Initial velocities were calculated for the first 10% of the reaction or less, and first-order rate constants were evaluated from the data comprising three half-lives of the reaction progress curve. Digital data were obtained using the macro Savetrace (Hewlett Packard).

In most of the studies evaluating the effects of monovalent cations on the hydrolysis of MTP, single kinetic runs were carried out (Figure 1). In experiments at pH 13 and above, the pH was held constant by use of the appropriate alkali metal hydroxide. Experiments were performed in triplicate only for potassium; the other rate constant determinations were single experiments. In the counterion studies at pH 7, MOPS buffers (25 mM) were made with the appropriate metal hydroxide. In studies at both pH 7 and pH 13, the total concentration of monovalent cation was varied using the chloride salt of the desired metal ion.

In the initial experiments on the effects of added divalent metal ions on the hydrolysis of MTP (Table II), the chloride salt of the metal was added to MTP at an approximately equal concentration. In kinetic titrations of the effects of variable concentrations of divalent metal ions (Table III), initial velocities of the hydrolysis were measured for solutions containing from approximately 20% to 90% of the metal–MTP. The titrations were carried out at 15.5 °C for $MgCl_2$ and 14.2 °C for $CoCl_2$ and $MnCl_2$ in 20 mM EPPS buffer at pH 8.0 and $I = 0.1$ with KCl. The total MTP concentration was 105 μM. These initial velocities were then fitted to

$$v_S - v_{exp} = (v_S - v_{LS})\frac{L_t + S_t + K_d - \sqrt{(L_t + S_t + K_d)^2 - 4L_tS_t}}{2S_t} \quad (1)$$

using a Fortran program and the least-squares method. In eq 1, v_S is the initial velocity for free MTP, v_{exp} is the experimentally measured velocity at a given metal concentration, v_{LS} is the velocity for the metal–MTP complex, L is the metal concentration, S is the MTP concentration, and subscript t denotes total concentration of a solute. Rate constants were calculated from initial velocities, and MTP concentrations were calculated from A_{227} using the apparent absorptivity of 3020 M^{-1} cm^{-1}, which appeared to be fairly constant regardless of conditions.

To verify that the foregoing kinetic titrations of divalent metal binding to MTP were not pH-dependent, the effect of pH on the rate of hydrolysis was evaluated between pH 6.8 and 8.0 at 25 °C, using HEPES buffer at an MTP concentration of 71 μM. Two rates were measured at each pH, with the metal ion concentration in the second run being 2.5 times that of the first. The two rates were used to calculate the difference in saturation using

$$\Delta(\%sat) = 100(v_1 - v_2)/(v_S - v_2) \quad (2)$$

in which v_1 is the initial velocity of the run with the lower metal concentration and v_2 is the initial velocity at the higher metal concentration. The basis for this equation is that the total MTP concentration is the sum of the free MTP and metal–MTP complex and that the rate of hydrolysis for the metal–MTP complex, v_2, is much smaller than that for MTP, v_S, or that v_2 is approximately the same as the rate for the metal–MTP complex. Any runs with greater than 5% difference (less than ~96% saturated for the second run) were rejected. The stability of thiophosphate produced in MTP hydrolysis was evaluated at pH 6.8 and 8.0 with 68 μM Na_3SPO_3 and metal concentration the same as the highest used in the MTP hydrolysis. The loss of A_{227} over time owing to thiophosphate decomposition was too small to interfere significantly with the rate determinations.

Activation Parameters for Hydrolysis of Metal–MTP Complexes. The temperature dependence for MgMTP hydrolysis was measured at temperatures ranging from 14.3 to 34.7 °C. The rates were measured in duplicate with the higher $[Mg^{2+}]$ at 2.11 mM and [MTP] at 75.6 μM. MTP was 98.5% saturated, as evaluated by its kinetically determined dissociation constant. The fits of data to eq 2 showed that the degree of saturation did not vary with temperature. The initial velocities were corrected to correspond to 100% saturation. The Arrhenius activation energies (E_a) were calculated from the slopes of the graphs of ln (k) vs $1/T$ (K^{-1}). The enthalpies of activation ($\Delta H^{\ddagger}$) were calculated from the slopes of the graphs of ln (k/T) versus $1/T$ (K^{-1}) (Bunnett, 1986). The entropies of activation were calculated by inserting the average rate constants at 25 °C into the equation $\Delta S^{\ddagger}/4.576 = \log(k_{avg}) - 10.753 - \log(T) + E_a/4.576T$, where T is the temperature in kelvin and $\Delta S^{\ddagger}$ is the entropy of activation (Bunnett, 1986). The parameters were calculated from least-squares fits of the rate constants collected at various temperatures.

The activation parameters for the hydrolysis of $[(CH_3)_4N]_4MTP$ were determined from the initial velocities of MTP hydrolysis between 40.6 and 55.7 °C at 63 μM MTP. The inital velocities were measured in duplicate at pH 12.2. The stability of Na_3SPO_3 was verified under the conditions of the rate measurements. A rate was also calculated from the study of Li_4MTP hydrolysis with 0.1 M $(CH_3)_4NOH$ at 25.0 °C and corrected for inhibition by 2.1 mM Li^+. The initial velocities and this calculated rate were then used to evaluate the activation parameters.

Magnetic Resonance Methods. NMR titrations of metal–MTP complexation at 5.0 °C and pH 12.5–13.5 were carried out using either ^{31}P or ^7Li NMR at 202.5 and 194.4 MHz, respectively, in 10-mm tubes. The lock signal was provided by a coaxial insert tube. The samples were 2 mL of 3 mM O_3PXPO_3 containing 0.2 M $(CH_3)_4NCl$–0.03 M $(CH_3)_4NOH$, where X = S, O, or CH_2. The LiCl titrations involved the addition of small volumes of a LiCl solution to the sample, so that the total volume changed less than 10%. The ^7Li shifts were compared to that of LiCl in deuteriomethanol/deuterioacetone in the insert tube to correct for any

field drift. In the KCl and NaCl titrations, the NMR sample was poured into an Eppendorf tube that contained a known amount of solid KCl or NaCl. This was mixed thoroughly and returned to the NMR tube. The chemical shifts of the contaminating phosphate and thiophosphate in the MTP samples changed with salt concentration as well. The integrations allowed the relative concentrations of species to be determined and fitted to appropriate equations. The change in chemical shift was then fitted to an equation related to eq 1, in which values of δ_S and δ_{exp} replaced v_S and v_{exp}, respectively; δ_S was the chemical shift of $[(CH_3)_4N]_4P_2O_6X$, and δ_{exp} was the chemical shift seen with a particular amount of monovalent metal chloride added. These fits were unsatisfactory in a number of cases in which the binding of a second metal ion perturbed the data. In these cases, the data were fitted to

$$\log (\delta_{exp} - \delta_S) = \log [(\delta_{LS} - \delta_S)L_t/(K_d + L_t) + PL_t] \quad (3)$$

in which P is the slope of the linear relationship. This equation was derived on the assumption of a hyperbolic relationship being superimposed upon a linear relationship, and K_d was taken as the dissociation constant for the first metal ion to bind.

EPR titrations of MTP were done at 16.5 °C in 10 mM EPPS and 100 mM KCl at pH 8.1. These experiments were done with 42 μM $MnCl_2$ and 25 μM Li_4MTP, giving 78% saturation. The solutions contained 0–146 μM $MgCl_2$ or 0–116 μM $CoCl_2$. The signal intensity for Mn^{2+} was calculated by adding together the peak heights of all six peaks. The increases in the concentrations of free Mn^{2+} upon displacement by Mg^{2+} or Co^{2+} were calculated from the enhancement of the signal and correlation with a standard curve generated with free Mn^{2+}. The data were fitted to

$$K_{11} = K_1[N_f^3 + N_f^2(K_1 - 2N_t - M_t + S_t) + N_fN_t(N_t - 2K_1 + M_t - S_t) + N_t^2K_1] / N_f^3 + N_f^2(K_1 + S_t - N_t) - N_fN_tK_1 \quad (4)$$

$$K_{11} = M_fS_f/MS \qquad K_1 = N_fS_f/NS = 5.4\ \mu M$$

where f = free; t = total; $N = [Mn^{2+}]$; $M = [Mg^{2+}]$ or $[Co^{2+}]$; and S = [MTP]. This equation was derived on the assumption of three species of MTP present in solution: S_f, NS, and MS. K_1 was the kinetically determined K_d for MnMTP, which could not be reliably measured in direct EPR experiments because of unacceptably fast hydrolysis of MTP.

The experiment with PP_i was done similarly, except that the solutions contained 8.4 μM $Li_4P_2O_7$, 100 mM KCl, 10 mM EPPS at pH 8.2, and 2.8–42.5 μM $MnCl_2$ at 15.2 °C. The concentration of free Mn^{2+} was calculated from the standard curve and then used to determine the dissociation constant in each experiment. These results were then averaged. A Scatchard plot of the data produced similar results.

Results

Interactions of Monovalent Metal Cations and MTP. Monovalent cations exert variable kinetic effects on the hydrolysis of MTP, as shown in Figure 1. At pH 7 and 13 LiCl inhibits hydrolysis, whereas NaCl, KCl, and CsCl enhance the rate of hydrolysis. The effect is wholly attributable to the cation and independent of the anion, on the basis of other experiments with alternate alkali metal salts (data not shown).

Monovalent cations can form complexes with alkyl phosphates, phosphate, and polyphosphates (Lambert & Watters, 1957; Watanabe et al., 1981; van Lier et al., 1983), and it is reasonable for the complexes with MTP to exhibit hydrolysis rates that differ from those for MTP itself. MTP can in principle bind up to four monovalent cations, and it is not difficult to observe the binding of one metal ion in thermodynamic experiments by observing the change in the ^{31}P NMR chemical shift for MTP. At high concentrations of metal chlorides, evidence of a second metal binding step is observed as a poor fit to a single binding isotherm. Equation 1 corresponds to a model of a single binding isotherm, whereas eq 3 assumes that there is one saturable change in chemical shift, plus a linear change resulting from a weak second interaction that does not approach saturation under the experimental conditions.

Table I: Dissociation Constants for Alkali Metal Complexes with MTP

substrate	titrant	K_d (mM)[a]
$^{2-}O_3PSPO_3^{2-}$	LiCl[b,c]	5.1 ± 1.2[d]
$^{2-}O_3PSPO_3^{2-}$	LiCl[e]	5.24 ± 0.04[d]
$^{2-}O_3PSPO_3^{2-}$	NaCl	107 ± 3[e]
$^{2-}O_3PSPO_3^{2-}$	KCl	84 ± 12[e]
$^{2-}O_3POPO_3^{2-}$	LiCl[b,c]	6.9 ± 1.2[d]
$^{2-}O_3POPO_3^{2-}$	LiCl	12.4 ± 0.2[e]
$^{2-}O_3POPO_3^{2-}$	NaCl	122 ± 3[e]
$^{2-}O_3POPO_3^{2-}$	KCl	156 ± 2[e]
$^{2-}O_3PCH_2PO_3^{2-}$	LiCl[e]	6.25 ± 0.13[e]
$^{2-}O_3PCH_2PO_3^{2-}$	NaCl	100 ± 5[e]
$^{2-}O_3PCH_2PO_3^{2-}$	KCl	126 ± 3[d]
PO_4^{3-}	NaCl	600 ± 50[d]
PO_4^{3-}	KCl	420 ± 30[d]
PSO_3^{3-}	NaCl	123 ± 8[d]
PSO_3^{3-}	KCl	300 ± 200[d]

[a] Experiments were single runs at pH 12.5–13.5 and 5.0 °C. The nucleus observed was ^{31}P at 202.5 MHz unless otherwise specified (footnote[b]), and the highest concentration of metal chloride in the titration was 1.6 M unless otherwise indicated (footnote *c*). [b] The nucleus observed was ^7Li. [c] The highest concentration of metal chloride was ≤30 mM. [d] Fits to eq 1. [e] Fits to eq 3. Only K_d for the first metal ion bound is given by the equation.

Table II: Effects of Divalent Metal Chlorides on the Hydrolysis of MTP

metal chloride	k_{obs} (min^{-1})[a]	metal chloride	k_{obs} (min^{-1})[a]
none	1.48	$NiCl_2$	0.78
$CuCl_2$	1.39	$CoCl_2$	0.60
$ClCl_2$	1.34	$MnCl_2$	0.39
$CdCl_2$	1.11	$ZnCl_2$	0.35
$MgCl_2$	1.07		

[a] Rate constants were measured in triplicate at 25 °C in 25 mM MOPS buffer at pH 7.0, with the ionic strength adjusted to 0.1 by addition of KCl. The concentration of MTP was 70 μM, and the metal chlorides were added in approximately equimolar concentrations. The observed first-order rate constants listed are the mean values, and the standard deviations were ≤10%.

The dissociation constants K_d for several alkali metal complexes of MTP, PP_i, methylenediphosphonate, and other complexes are given in Table I. The values of K_d for $LiMTP^{3-}$ determined by ^7Li NMR and ^{31}P NMR are in agreement; they are all 5–12 mM, showing that PP_i and its analogues bind Li^+ tightly. This explains the nearly constant inhibition of hydrolysis in Figure 1B, where all the Li^+ concentrations are well above K_d. The values of K_d for Na^+ and K^+ are an order of magnitude larger, ranging from 84 mM for $KMTP^{3-}$ to 156 mM for KPP_i^{3-}. The K_d values for alkali metal complexes with phosphate and thiophosphate trianions are 123–600 mM.

All titrations of MTP, PP_i, and methylenediphosphonate that were evaluated at metal chloride concentrations as high as 1.7 M gave evidence of a secondary metal dependence, except for potassium methylenediphosphonate.

Interactions of Divalent Metal Ions with MTP. The data in Table II show that divalent metal ions equimolar with MTP inhibit the hydrolysis at pH 7, no doubt as a result of complex formation between MTP and divalent metal cations. The

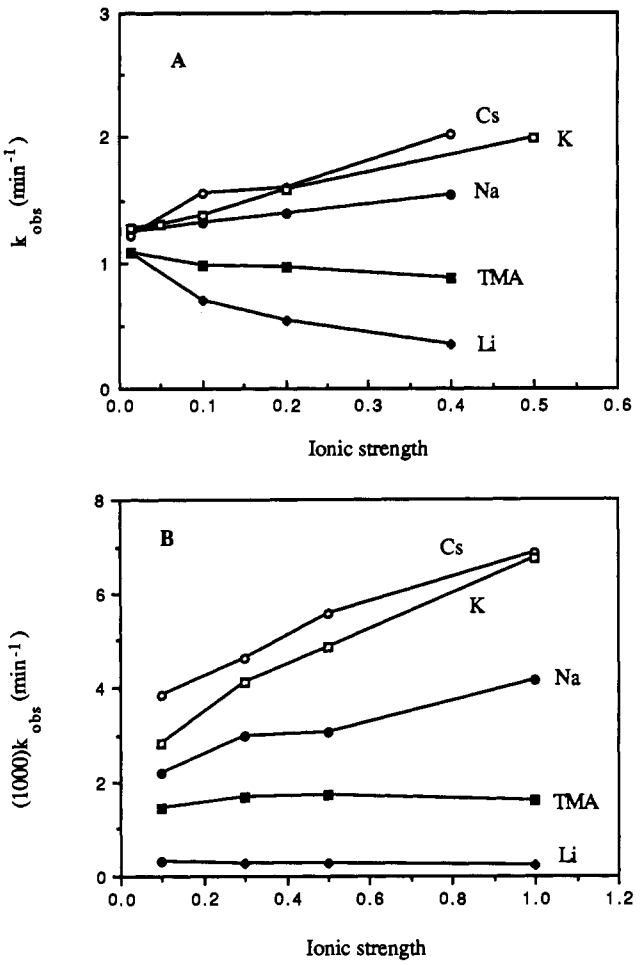


FIGURE 1: Effect of alkali metal chlorides on the rate constant for MTP hydrolysis. Part A: The reaction mixtures contained the alkali metal salts of MOPS buffer at 25 mM and pH 7.0, with the metal chlorides added at concentrations that gave overall ionic strengths of 0.014–0.4. Points represent single runs except for potassium, where they represent the average of three determinations. Part B: The reaction mixtures were prepared at pH 13 with the alkali metal hydroxides supplemented with the corresponding chlorides to give the desired ionic strengths. Points represent single runs.

divalent metal–MTP complexes ($MeMTP^{2-}$) are less reactive than $HMTP^{3-}$ at pH 7 because of the loss of the proton associated with the leaving group. The metal in the complex depresses the fourth pK_a of MTP, with the result that MTP exists as a mixture of $HMTP^{3-}$ + $MeMTP^{2-}$ at pH 7, and the latter is less reactive. Therefore, the positive charges contributed by the metals in the complexes do not compensate for the absence of a covalent bond to a proton.

The effects of metal ions on the hydrolysis of MTP allowed the K_d values for complexes to be evaluated by rate measurements. In a simple dissociation process, the observed rate will be the sum of the rates for free MTP and metal–MTP. The rates vary with the concentration of metal chloride, from that for $HMTP^{3-}$ to the rate for $MeMTP^{2-}$ at saturating metal chloride. The data can be fitted to a binding equation to evaluate the K_d for the complex.

Kinetically evaluated dissociation constants for several transition-metal complexes at pH 8.0 are presented in Table III. The values of $K_{d\,app}$(pH 8.0) listed are those determined by fitting rates to eq 1. This procedure also evaluates the rates of hydrolysis of the metal–MTP complexes, which turn out to be insignificant relative to the rate of MTP hydrolysis at pH 8.0, where the dominant form is $HMTP^{3-}$. Therefore, the inhibitory effects of Mg^{2+}, Mn^{2+}, and Co^{2+} in Table II result

Table III: Kinetically and Thermodynamically Determined K_d Values for Divalent Metal–MTP Complexes[a]

complex	temp (°C)	$K_{d\,app}$(pH 8.0) (μM)	K_d(calc) (μM)
	Kinetic Values		
MgMTP	15	32 ± 4	7.7 ● 10
MnMTP	14	5.4 ± 1.4	1.3 ± 0.3
CoMTP	14	27 ± 15	6.5 ± 3.6
	Thermodynamic Values		
$MnPP_i$	16	3.4 ± 0.5	0.97 ● 0.14
MgMTP	16	28 ± 13	6.7 ● 3.1
CoMTP	16	11 ± 4	2.6 ± 0.9

[a] The effects of varying divalent metal chloride concentrations on the rate of MTP hydrolysis were determined at pH 8.0. The rate differences owing to the inhibitory effect of the divalent metal ions were fitted to eq 1 in order to evaluate apparent K_d at pH 8.0 [$K_{d\,app}$(pH 8.0)]. The dissociation constants for the complexes [K_d(calc)] were calculated from the apparent values at pH 8.0 by using the value 3.16 × 10^{-9} M as the acid dissociation constant for $HMTP^{3-}$ (pK_a = 8.49; Halkides & Frey, 1991). The thermodynamic values were obtained in EPR titration experiments as described under Experimental Procedures.

mainly from the formation of transition metal–MTP complexes.

Thermodynamic values of the dissociation constants for the complexes of Mn^{2+}, Mg^{2+}, and Co^{2+} with PP_i and MTP are in agreement with the kinetically determined values for MTP complexes in Table III. Thermodynamic values are derived from EPR measurements of the effects of complexation on the EPR signals for Mn^{2+}; complexation by Mg^{2+} and Co^{2+} is observable as competition with Mn^{2+}. Note that the thermodynamically measured value of $K_{d\,app}$(pH 8.0) for $MnPP_i^{2-}$ is the same within experimental error as the kinetically determined value for $MnMTP^{2-}$ in Table III. The thermodynamic values for $MgMTP^{2-}$ and $CoMTP^{2-}$ are also within experimental error of the kinetically determined values in Table III. Neither the kinetic nor the thermodynamic determinations of K_d reveal any evidence of the formation of bis-metal complexes under the conditions of the experiments.

All available evidence indicates that complexation of divalent metal ions by MTP is similar to that by PP_i. The values of K_d(calc) are in the range of 1–7.7 μM for complexation of divalent metals ions by MTP^{4-}. When compared with the dissociation constants for monovalent metal ions in Table II, the values are smaller by factors of over 1000-fold.

Hydrolytic Rate Constants and Activation Parameters for MTP^{4-} and Metal–MTP Complexes. First-order rate constants for the hydrolysis of MTP^{4-}, $HMTP^{3-}$, and various metal–MTP complexes in the temperature range of 5–35 °C are given in Table IV. Complexation of MTP^{4-} with Na^+, Cs^+, K^+, and Mg^{2+} enhances the hydrolytic rate by factors of 1.8–3 at 25 °C. Li^+ inhibits the hydrolysis by a factor of 8. At 35 °C, Co^{2+} enhances the hydrolytic rate 2-fold and Mn^{2+} inhibits by a factor of 1.7. These are very modest effects. The principal conclusion from these measurements is that metal complexation moderately perturbs the hydrolysis of MTP^{4-}.

The data in Tables II and III show that divalent cations severely retard the hydrolysis of MTP at pH 7 and 8, where the predominant form ($HMTP^{3-}$) is converted by complexation to the complexes metal–MTP^{2-} and a proton. Inhibition of hydrolysis is brought about largely by the perturbation in the pK_a of $HMTP^{3-}$ through metal complexation. In contrast, Figure 1A shows that several monovalent cations enhance the hydrolysis rates at pH 7.

The activation parameters for MTP^{4-} and $MgMTP^{2-}$ are compared in Table V with those for $HMTP^{3-}$ reported in another paper (Halkides & Frey, 1991). These values for ΔS^*

Table IV: First-Order Rate Constants for the Hydrolysis of Various Species of MTP

species	temp (°C)	$k \times 10^3$ (min^{-1})	species	temp (°C)	$k \times 10^3$ (min^{-1})
MTP^{4-}	15	0.46[a]	$MnMTP^{2-}$	14	<2
MTP^{4-}	25	2.1	$MnMTP^{2-}$	25	<4.3
MTP^{4-}	35	11.2	$MnMTP^{2-}$	35	6.4[c]
MTP^{4-}	40	25	$CoMTP^{2-}$	14	<11
MTP^{4-}	49	96	$CoMTP^{2-}$	26	<5.9
$HMTP^{b}$	15	412	$CoMTP^{2-}$	35	23[c]
$HMTP^{b}$	25	1350	$LiMTP^{3-}$	25	0.25[d]
$HMTP^{b}$	35	4110	$NaMTP^{3-}$	25	3.9[d]
$MgMTP^{2-}$	15	1.8	$KMTP^{3-}$	25	6.7[d]
$MgMTP^{2-}$	20	3.6	$CsMTP^{3-}$	25	6.7[d]
$MgMTP^{2-}$	25	5.9[a]			
$MgMTP^{2-}$	35	16			

[a] Value calculated using the activation energy and the Arrhenius equation. [b] Data from Halkides and Frey (1991). Rates were measured at pH 7.0 and probably correspond to hydrolysis of the trianion ($HMTP^{3-}$). [c] Single determinations. [d] Estimated by extrapolation of data in Figure 1B to saturating metal ion. The error in these extrapolations is estimated at ±20%.

Table V: Activation Parameters for the Hydrolysis of Various Species of MTP

MTP species	$\Delta H^{\ddagger}$ (kcal·mol^{-1})	$\Delta S^{\ddagger}$ (cal·mol^{-1}·deg^{-1})
MTP^{4-}	28	14
$HMTP^{3-}$	20	0.2
$MgMTP^{2-}$	19	−12

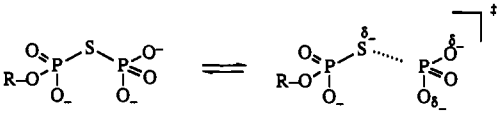
are within the range generally observed for reactions that proceed through dissociative transition states. There are substantial differences, however, which signify the problems associated with the interpretation of activation parameters in mechanistic terms. The values of $\Delta S^{\ddagger}$ vary from −12 eu to +14 eu, depending on the overall charge of the reacting species. It seems reasonable for the solvation of MTP^{4-}, $HMTP^{3-}$, and $MgMTP^{2-}$ to vary in both the ground state and the transition state for hydrolysis, and it is possible that these differences are important factors in determining the activation parameters.

The activation energies for the hydrolysis of $MgMTP^{2-}$ and MTP^{4-} allow us to calculate the rate constant 5.3×10^{-4} min^{-1} and 7.7×10^{-5} min^{-1}, respectively, at 5 °C. The value for $MgMTP^{2-}$ is important for understanding the phosphoryl group transfer reactivity of $MgMTP^{2-}$ at the active sites of pyrophosphate dependent phosphofructokinase at 5 °C, which is reported in an accompanying paper (Halkides et al., 1991).

Discussion

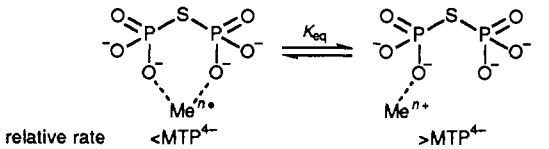
The K_d values for the metal ion–MTP complexes reported herein are similar to those for comparable metal ion–PP_i complexes reported in this paper and in the literature (Lambert & Watters, 1957; Delannoy et al., 1979; Frey & Stuehr, 1972; Melardi et al, 1979; Irani, 1961; Cooperman & Mark, 1971). The effects of metal ions on the hydrolysis of PP_i are analogous to those we observe on the hydrolysis of MTP. In particular, Watanabe et al. (1981) observed Li^+ retardation of PP_i hydrolysis at pH 5 and 7, whereas alkali metals generally enhance phosphoryl-transfer rates (Watanabe et al., 1981; Hopkins & Wang, 1965). Although metal ions perturb the hydrolytic rates for MTP and PP_i to similar degrees, the hydrolytic rates for metal ion–MTP complexes are millions of times faster than those for analogous metal ion–PP_i complexes.

Two metal ions studied in this research, Li^+ and Mn^{2+}, retard the hydrolysis of MTP^{4-} whereas the other metals increase the hydrolytic rate. We are unable to account for this difference with certainty. However, we can offer a rationale that seems to be consistent with the MTP-binding properties of Li^+ and Mn^{2+} and the known chemistry of phosphoryl transfer. The effects of metal ions on phosphoryl transfer are not obviously predictable; however, it seems that metal ion complexation to the phosphoryl group undergoing transfer would be unlikely to facilitate the reaction through a dissociative transition state such as that for MTP hydrolysis. This is because complexation to the transferring phosphoryl group would reduce its negative charge in the ground state, and dual negative charges are a required driving force for reaching a dissociative transition state as shown:



where the substituent R can be a proton or an alkyl group. Metal ion complexation of the incipient metaphosphate would tend to retard the reaction by stabilizing the charge in the ground state (Knowles, 1980). MTP binds Li^+ more strongly than other alkali metals, and it also binds Mn^{2+} more strongly than other divalent cations we have studied (Tables I and III). It may be that Li^+ and Mn^{2+} form more stable bidentate complexes with MTP than the other related cations we have studied.

There is little doubt that the complexes of divalent metal ions with MTP^{4-} are largely bidentate, and it may be that the Li^+ complex is as well. In a symmetrical bidentate complex, the negative charge on both phosphoryl groups is reduced by the metal, and this should reduce the rate of cleavage. In a monodentate complex, however, one phosphoryl group carries two charges and the other only one, and this should facilitate the reaction, with the metal ion stabilizing the leaving group as does the group R shown above. A symmetrical bidentate metal–MTP complex should be in equilibrium with a monodentate complex according to eq 5. Complexes for which K_{eq}



relative rate <MTP^{4-} >MTP^{4-} (5)

is very small will exist largely in the form of the symmetrical bidentate complex on the left, which is less reactive than MTP^{4-}. Such complexes are likely to be those with the smallest values for K_d, the metal dissociation constants; this may explain why Li^+ and Mn^{2+} inhibit the hydrolysis of MTP^{4-}. In the case of Na^+, K^+, Cs^+, Mg^{2+}, and Co^{2+}, the values of K_{eq} may be large enough to allow a significant fraction of the monodentate complex to exist at equilibrium; the greater reactivity of the monodentate complexes relative to MTP^{4-} can allow the metal to enhance the observed rate. The complexes of MTP with Na^+, K^+, and Cs^+ may or may not be mainly monodentate, but those with Mg^{2+} and Co^{2+} are most likely largely bidentate at equilibrium.

The data in Tables II and III show that divalent cations severely retard the hydrolysis of MTP at pH 7 and 8. This effect is caused by the reaction of the predominant species $HMTP^{3-}$ with a divalent metal ion to give $MeMTP^{2-}$ with release of H^+. That is, complexation with a divalent metal ion lowers the fourth pK_a of MTP. Hydrolysis is inhibited because the species $MeMTP^{2-}$ are much less reactive than $HMTP^{3-}$. Data for PP_i indicate that complexation with divalent metal ions lowers the fourth pK_a from 8.36 to 6.02 (Frey & Steuhr, 1972). A similar lowering of pK_a can be expected for $HMTP^{3-}$, and this interpretation is supported by the fact that hydrolysis is pH-independent between pH 6.8 and 8 in the presence of divalent metal ions. Therefore, the inhibition

of hydrolysis by divalent metal ions at pH 8 is caused by a shift in the pH–rate profile for the divalent metal–MTP complexes to the left of that for MTP.

In contrast to the inhibition by divalent metals at pH 7 and 8, Figure 1 shows that several monovalent cations enhance the hydrolysis rates at pH 7. Moreover, the inhibition by Li^+ is very modest compared with the effects of the divalent metals. The data of Lambert and Watters (1957), who carefully titrated PP_i in the presence and absence of alkali metal ions, show that pK_a perturbations by monovalent cations are much smaller than those by divalent metal ions; there is no effect of Na^+, K^+, and Cs^+ on the PP_i charge type at pH 7. Therefore, complexation of HPP_i^{3-} by these ions at pH 7 does not lead to deprotonation, and the metal complexes exhibit increased reactivity. Lambert and Watters also showed that Li^+ complexation induces larger pK_a perturbations than complexation by the other alkali metal ions, and Li^+ significantly alters the charge type at pH 7. This effect, which may result from a tendency for the Li–MTP complex to be bidentate, accounts for inhibition by Li^+ at pH 7.

The entropies of activation for the hydrolysis of MTP species in Table V indicate that the transition state for the hydrolysis of MTP^{4-} is more disordered than the ground state. The rate changes by a fairly unusual 5.2-fold per 10-deg change in temperature. In the hydrolysis of $HMTP^{3-}$, little change in entropy occurs in going from the ground state to the transition state. The transition state for the hydrolysis of $MgMTP^{2-}$ is somewhat less disordered than the ground state. The *differences* in $\Delta S^{\ddagger}$ probably signal differences in solvation in the ground states and transition states for these species. The charge differences among the three species can be expected to lead to differences in solvation. None of the values corresponds to what would be expected for a kinetically bimolecular process. The larger enthalpy of activation for the tetraanion compared with the other two species may result from the fact that the leaving group (SPO_3^{3-}) is poorer than those in the reactions of the other two species, and bond cleavage to the leaving group may be less advanced in the transition state.

Effects of Metal Ion Complexation on the Mechanism of Phosphoryl Group Transfer. The effects of metal ion complexation on the rate at of MTP hydrolysis are very small compared with rate enhancements at enzymic active sites, and some metal ions inhibit hydrolysis. The results do not support the view that metal ion complexation at active sites is an important catalytic mechanism in phosphoryl group transfer, although metal complexation is certainly important for neutralizing charges and facilitating binding. It is clear that specific acid catalysis through $HMTP^{3-}$ is far more effective than metal complexation in promoting the hydrolysis of MTP^{4-}.

The rate constants in Table II do not follow a simple correlation with ionic radius of the metal ions (Nightingale, 1959) or the presumed stability constant based on the behavior of pyrophosphate (Karweik & Haber, 1978). Metal ions such as Cd^{2+}, Zn^{2+}, and Co^{2+} are expected to interact with sulfur to a greater extent than the other metals. The data provide no evidence that coordination between any of the metals and the bridging sulfur is important in the hydrolysis mechanism. The results are in this respect analogous to those of Dittmer et al. (1963) who investigated the hydrolysis of *S-n*-butyl phosphorothiolate.

The limited information on metal complexation in the hydrolysis of PP_i is analogous to our results for MTP (Watanabe, 1981; Hofstetter & Martell, 1959; Green, 1950). Most of the literature reports of metal catalysis in the hydrolysis of phosphoanhydrides pertains to the hydrolysis of nucleoside triphosphates such as ATP. The effects of the nucleotide substituents on hydrolysis are electronically similar to protonation, as in $HMTP^{3-}$ compared with MTP^{4-}, so that the reported effects are not directly comparable to our results. However, it is significant that in all of the studies of metal-catalyzed hydrolysis of nucleoside triphosphates the most effective metals were Zn^{2+}, Cu^{2+}, Ni^{2+}, and trivalent metals, whereas Mg^{2+}, the biologically most significant metal for phosphoryl group transfer, was essentially ineffective. The most catalytically active metal ions increase the rates of hydrolysis by factors of 5–60 in the pH range of 5–9 (Tetas & Lowenstein, 1963), and these rate enhancements are compounded by complexation with a second metal ion (Sigel & Amsler, 1976; Milburn et al., 1985).

Mg^{2+} inhibits phosphoryl group transfer from *N*-phosphorylimidazoles and *N*-phosphorylpyridines, and metal ions do not catalyze dephosphorylation of phosphorylguanidines (Lloyd et al., 1971; Prigodich & Haake, 1984; Herschlag & Jencks, 1987). In general, metal ions do not catalyze dephosphorylation of phosphoramidates with neutral leaving groups; however, dephosphorylation of *p*-nitrophenyl phosphate by substituted pyridines is catalyzed by Mg^{2+} and Ca^{2+} (Herschlag & Jencks, 1987). It has been suggested that metal ion catalysis may be most important when the leaving group is negatively charged and coordination with a metal in the transition state promotes its departure (Hershlag & Jencks, 1987). This interpretation can also explain metal ion catalysis of the nucleophilic cleavage of phosphoanhydrides, as explained above for MTP.

Herschlag and Jencks (1987) definitively showed that divalent metal ions do not significantly perturb the mechanisms or transition states for phosphoryl transfer from *N*-phosphorylpyridines, *p*-nitrophenyl phosphate, or 2,4-dinitrophenyl phosphate. Metal ion coordination modestly enhanced or inhibited these reactions; however, the linear free energy correlations clearly showed that the transition state remained dissociative in character when divalent metals were coordinated to the transferring phosphoryl groups.

Acknowledgments

This study made use of the National Magnetic Resonance Facility at Madison, which is supported in part by NIH Grant RR02301 from the Biomedical Research Technology Program, Division of Research Resources. Equipment in the facility was purchased with funds from this program, the University of Wisconsin, the NSF Biological Instrumentation Program (Gran DMB-8415048), the NIH Shared Instrumentation Program (Grant RR02781), and the U.S. Department of Agriculture. We thank Professor W. W. Cleland, for writing the computer programs to fit our data to equation, and G. H. Reed for guidance in EPR experiments.

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The Substrate Reactivity of μ-Monothiopyrophosphate with Pyrophosphate-Dependent Phosphofructokinase: Evidence for a Dissociative Transition State in Enzymatic Phosphoryl Group Transfer†

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Received June 10, 1991; Revised Manuscript Received July 30, 1991

ABSTRACT: μ-Monothiopyrophosphate (MTP), an analogue of pyrophosphate (PP_i) with sulfur in place of oxygen in the bridge position, is a substrate for the enzyme pyrophosphate-dependent phosphofructokinase. At pH 9.4 and 6 °C, the maximal velocity for the phosphorylation of fructose 6-phosphate (F6P) by MgMTP is about 2.8% of that with $MgPP_i$ as the phosphoryl donor. The kinetic mechanism is equilibrium random with rate-limiting transformation of the substrate ternary complex to the product when either MgMTP or $MgPP_i$ is the phosphoryl donor. This is known from independent studies to be the kinetic mechanism at pH 8.0 and 25 °C [Bertagnolli, B. L., & Cook, P. F. (1984) *Biochemistry 23*, 4101-4108]. The dissociation constant of $MgPP_i$ is 14 μM, that of MgMTP is 64 μM, and that of F6P from the enzyme is about 5 mM. The K_m values for $MgPP_i$ and MgMTP are 14.5 and 173 μM, respectively. MgMTP competes with $MgPP_i$ for binding to the enzyme. The values of k_{cat} are 3.4 s^{-1} and 140 s^{-1} for MgMTP and $MgPP_i$, respectively, at pH 9.4 and 6 °C. The estimated rate enhancement factors are 3.6×10^5 and 1.4×10^{14} for the reactions of MgMTP and $MgPP_i$, respectively. Therefore, MgMTP is a reasonably good substrate for PP_i-dependent PFK, on the basis of comparisons of k_{cat}. However, the rate enhancement factors show that the enzyme is a poor catalyst for the reaction of MgMTP. Lesser enzymatic catalysis in the reaction of MgMTP compared with $MgPP_i$ is largely compensated for by the greater intrinsic reactivity of MgMTP. Thus, the larger substrate MgMTP is well accommodated in the active site, and the dissociative reaction of MgMTP is well accommodated in the transition state. The results are interpreted to indicate a dissociative transition state for phosphoryl group transfer by PP_i-dependent PFK. A modified synthesis and purification of MTP are described, in which (trimethylsilyl)trifluoromethanesulfonate and tetra-*N*-butylammonium iodide are used in place of iodotrimethylsilane to dealkylate tetramethyl-MTP.

Phosphoryl group transfers are among the most common and important reactions in biochemistry. Despite this fact, the chemical mechanism of enzymatic phosphoryl group transfer is poorly understood (Knowles, 1980). In particular, the nature of the transition state is not known.

Nonenzymatic phosphoryl group transfer reactions in protic solvents proceed through dissociative transition states, in which there is little bonding between the phosphoryl group in flight and either the leaving group or the attacking nucleophile (Bunton, 1970; Benkovic & Schray, 1973; Williams, 1989;

† This research was supported by Grant GM30480 to P.A.F. from the National Institute of General Medical Sciences and by a predoctoral fellowship to C.J.H. from the National Science Foundation.

0006-2960/91/0430-10313$02.50/0