Metal Ion and Nucleophilic Catalysis of Pyridine-2-carbaldoximyl Phosphate Hydrolysis

Chih-Min Hsu and Barry S. Cooperman*

Contribution from the Department of Chemistry, University of Pennsylvania, Philadelphia, Pennsylvania 19174. *Received November 3, 1975*

Abstract: Pyridine-2-carbaldoximyl phosphate was synthesized, characterized, and found to form a complex with Zn^{2+}, the dianion form having an association constant for Zn^{2+}, at 25 °C, of 790 M^{-1}. Toward both hydrolysis and nucleophilic catalysis of hydrolysis strong evidence was obtained that the Zn^{2+} complex behaved as a typical phosphate monoester and that the catalytic effects of Zn^{2+} in both reactions could be accounted for strictly in terms of leaving-group activation. The observed catalytic effects were compared to the maximum catalysis attainable via leaving-group activation by metal ions, as calculated from the Bronsted leaving-group β's for the hydrolysis and nucleophilic reactions of phosphate monoesters and the acidities of divalent metal ions. These results, in combination with those in the accompanying paper,[2] were used to construct a model of the transition state for the nucleophilic attack reaction. In addition, the implications of this work for enzymatic catalysis of phosphoryl transfer were considered.

Introduction

We have previously shown[1] that the Zn^{2+}-pyridine-2-carbaldoxime (Zn^{2+}-PCA) anion can attack phosphoramidates via a ternary complex to give pyridine-2-carbaldoximyl phosphate (PCAP) and in this paper we present a synthetic method for producing this material in quantity and present evidence for its structural characterization. In an accompanying paper[2] we determined a Bronsted leaving-group β for Zn^{2+}-PCA attack on phosphoramidates derived from alkyl and aralkyl amines. However, use of this β value to arrive at mechanistic conclusions was subject to some uncertainty, since it was not entirely clear to what extent the rate constants used to determine β actually included both rate and equilibrium constants. Since nucleophilic attack by amines on Zn^{2+}-PCAP is the microscopic reverse of Zn^{2+}-PCA attack on phosphoramidates, we reasoned that the ambiguity in the leaving-group β value could be removed by determining a nucleophilic β value for amine attack on Zn^{2+}-PCAP, which is what we have done in the present paper, along with, as a necessary prerequisite, a study of the uncatalyzed hydrolysis of PCAP and Zn^{2+}-PCAP. These studies have led us to the conclusion that for both the hydrolysis and nucleophilic attack reactions, the structures of the transition states in the presence of Zn^{2+} are similar to those of analogous reactions which take place in the absence of metal ions.

Experimental Section

Materials and Equipment. PCAP was prepared as follows. To 10 mmol of Zn^{2+}-PCA in 250 ml of water was added 4.6 g (20 mmol) of calcium phosphorylimidazole. The solution was maintained at 40 °C and pH 6.0 for 30 min, adjusted to pH 1, 4.7 g (30 mmol) of bipyridine was added, and the entire reaction mixture was readjusted to pH 6.0 and lyophilized. The resulting solid was dissolved in the minimum amount of water and the solid formed on readjusting the solution to pH 6 was removed by filtration. The filtrate was applied to a Dowex 1 (Cl^-) column (19 × 300 mm, flow rate 0.5 ml/min) and the column was first washed with water to remove bipyridine and unreacted PCA and PIm (detected by monitoring the eluate at 233 and 280 nm), then PCAP was eluted with 0.1 N HCl. Pooled fractions containing PCAP were concentrated and adjusted to pH 3.0 with 0.1 N NaOH. The precipitate formed on standing at 0 °C was recrystallized from a water-ethanol mixture, yielding 0.6 g (30%) of zwitterionic PCAP. The crystalline product showed a single spot on paper chromatography (Whatman No. 40) (R_f 0.67, 0.1 M K_2CO_3-ethanol, 3.5:6.5) and TLC on polyethylenimine plates (Macherey-Nagel MN 300) (R_f 0.21, 2% LiCl). The phosphate analysis (giving mol wt 206 ± 4; calculated 202), uv spectrum [pH 13; λ_{max} 277 nm (ϵ 8.3 × 10^3); λ_{max} 240 nm (ϵ 10.2 × 10^3); virtually identical with that for pyridine-2-carbaldoxime (PCA) in the pH range 6-8[1]], ^1H NMR spectrum (also very similar to that found for PCA), and C/P analytical ratio (calcd 2.32; found 2.31) are all in accord with the presumed structure, whose molecular formula is $C_6H_7N_2O_4P$. PCAP was also prepared in an exactly analogous manner using phosphoramidate as a phosphoryl donor, with a yield of 35%. In some preparations, following the above procedure, crystals of the monosodium salt of PCAP ($C_6H_6N_2O_4P$·Na) were obtained in place of the PCAP zwitterion, as shown by potentiometric titration and from the molecular weight of 224 ± 4 determined by phosphate analysis.

anti-Pyridine-2-carbaldoxime,[3] calcium phosphorylimidazole,[1] and potassium ammonium phosphoramidate[4,5] were prepared as previously described. Pyridine (99.5%) was obtained from Matheson, Coleman, and Bell. All other pyridine derivatives were obtained from Aldrich. $NiCl_2·6H_2O$, $ZnSO_4·7H_2O$, KCl, and the disodium salt of succinic acid were Baker analyzed reagents.

Ultraviolet measurements were made on either a Cary 15 recording spectrophotometer or a Zeiss PMQII spectrophotometer. ^1H NMR spectra were taken on a Varian A60A spectrometer and ^{31}P NMR spectra were taken on a JOEL-PS-100 spectrometer. Potentiometric titrations were made with a Radiometer automatic titrator. Measurements of pH at elevated temperatures were made by a standard procedure.[6]

Kinetic Procedures. Rates of PCAP hydrolysis were measured either by phosphate appearance, using the modified Martin-Doty procedure of Jencks and Gilchrist,[7] or by measuring the increase in uv absorbance at 295 nm (or 305 nm) of reaction-mixture aliquots which are quenched in a NaOH (0.02 M)-Na_4EDTA (0.03 M) solution. The increase results from PCA anion formation. Reaction mixtures were brought to an ionic strength of 2.0 with KCl and were buffered with 0.2 M succinate. Reactions were generally followed for 2 half-lives, at least six points were taken per run, and all rate constants were measured at least twice. Reproducibility was ±5% for the phosphate assay and ±7% for the uv assay. Temperature variation was ±0.1 °C and pH variation during the course of a run was within ±0.15 pH units.

Results

PCAP and Zn^{2+}-PCAP Equilibria. The equilibria which are important for the interaction of Zn^{2+} with low concentrations (<0.5 mM) of PCAP in the pH range 5.0-6.5, along with their equilibrium constants, are listed in Table I. Values of K_1, K_3, and K_4 were determined spectrophotometrically, the latter two constants from a computerized analysis of data obtained by titration of PCAP with Zn^{2+} at several pH values in the range 5.5-6.5. Relevant spectral parameters are listed in Table II. K_2 was determined by ^{31}P NMR titration, the observed chemical shift rising from −5.30 ppm (relative to 85% H_3PO_4) for $PCAP^{2-}$ to −1.65 ppm for $PCAPH^-$. Such a large upfield shift on protonation is typical of phosphate esters.[8,9] K_4 is larger than the association constant for Zn^{2+} binding to

Table I. Equilibrium Constants[e]

Equilibrium		Constant	Method
$H_2PCAP \rightleftharpoons PCAPH^- + H^+$	K_1	1.5×10^{-4} M	*a,b*
$PCAPH^- \rightleftharpoons PCAP^{2-} + H^+$	K_2	1.5×10^{-6} M	*a,c*
$PCAPH^- + Zn^{2+} \rightleftharpoons Zn^{2+}$-PCAPH$^-$	K_3	326 ± 90 M^{-1}	*b*
$PCAP^{2-} + Zn^{2+} \rightleftharpoons Zn^{2+}$-$PCAP^{2-}$	K_4	790 ± 83 M^{-1}	*b*
Zn^{2+}-$PCAPH^- \rightleftharpoons Zn^{2+}$-$PCAP^{2-} + H^+$	K_5	3.6×10^{-6} M	*d*

[a] Potentiometric titration. [b] Spectrometric titration. [c] ^{31}P NMR titration. [d] As calculated from K_2K_4/K_3. [e] 25 °C, μ = 2, KCl.

Table II. Uv Spectral Data

	pH	λ_{max}, nm	$\epsilon_{max} \times 10^{-3}$
PCAP	1.5	240	8.5
		294	11.2
PCAP	6	240	8.1
		277	9.9
PCAP, 0.1 M Zn^{2+}	6	289	9.0

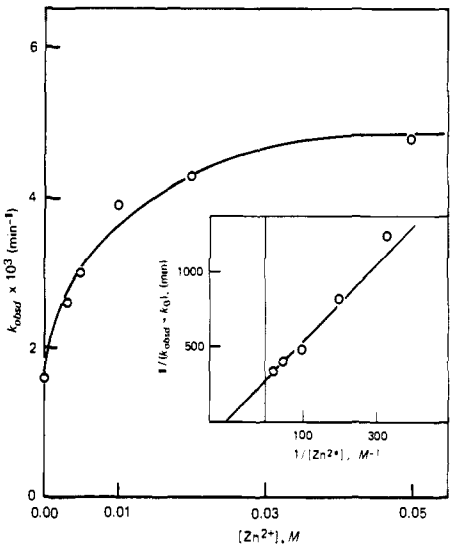

Figure 1. k_{obsd} (phosphate analysis) for PCAP hydrolysis as a function of Zn^{2+} concentration, pH 6.0, 80 °C, μ = 2 (KCl). Inset: $(1/k_{obsd} - k_o)$ vs. $1/[Zn^{2+}]$; k_o is k_{obsd} (phosphate analysis) in the absence of Zn^{2+}.

neutral pyridine-2-carbaldoxime (150 M^{-1}), which could be taken as evidence that PCAP functions as a tridentate ligand for Zn^{2+}, although it could also be argued that such an increase could arise from an attractive charge-charge interaction rather than via direct binding of Zn^{2+} to the phosphoryl moiety.

PCAP Hydrolysis. The rate of PCAP hydrolysis at pH 6, as measured by phosphate release, reaches a saturating value as a function of Zn^{2+} concentration with an apparent binding constant of 105 M^{-1} (Figure 1). This value is about a factor of six lower than the apparent binding constant obtained at 25 °C and pH 6 via spectrophotometric titration (605 M^{-1}), as would be expected for a complexing agent with two nitrogen ligands. At saturating Zn^{2+} levels, rates for PCAP hydrolysis as measured by PCA release using the uv method (see Experimental Section) were essentially identical with those measured by phosphate release. Thus, at saturating Zn^{2+}, the hydrolysis reaction is correctly written as

$$Zn^{2+}\text{-PCAP} \rightarrow Zn^{2+}\text{-PCA} + P_i \quad (1)$$

However, in the absence of Zn^{2+}, PCA release, as measured by increasing $A_{305\ nm}$, is some twofold slower than phosphate release. In fact, the true rate of PCA release may be even more than twofold slower if there are any other chromophores present in the reaction mixture which are derived from PCAP and have appreciable absorption at 305 nm. Moreover, ^1H NMR spectra of the reaction mixture clearly show the rate of PCAP disappearance to exceed the rate of PCA appearance. These results, taken together with the finding that PCA decomposes to only a minor extent after 24 h at pH 6 and 80 °C (compared to a half-life for phosphate release of 6.5 h), provide strong evidence that phosphate release results from other reactions in addition to

$$PCAP \rightarrow PCA + P_i \quad (2)$$

Two possibilities we have considered are: (1) that PCAP, synthesized in the syn form, isomerizes to *anti*-PCAP and hydrolyzes via intramolecular nucleophilic attack of the pyridine nitrogen; or (2) that PCAP hydrolyzes via N-O cleavage.[30] These possibilities can, however, be excluded since, despite a careful search, no evidence could be found for formation of either of the expected pyridine-containing products, *anti*-PCA or 2-cyanopyridine (or its hydrolysis product, 2-picolinamide). The assumptions that the increase in $A_{305\ nm}$ is due solely to PCA formation and that PCA arises solely from eq 2 permit calculation of an upper-limit rate constant for eq 2 of 0.85×10^{-3} min^{-1}.

Observed rate constants for Zn^{2+}-PCAP hydrolysis as a function of several variables are listed in Table III. If, by analogy to K_{app} at pH 6, we assume that K_3 and K_4 are also sixfold weaker at 80 °C than the measured values at 25 °C and furthermore that K_2 is unchanged (from the known temperature insensitivity of secondary phosphate pK_a's[10]), then from the values listed in Table I it is possible to show that at 80 °C, 0.05 M Zn^{2+}, and 0.2 mM PCAP, Zn^{2+}-$PCAP^{2-}$ and Zn^{2+}-PCAPH$^-$ are the dominant PCAP-containing species in solution, with Zn^{2+}-$PCAP^{2-}$ predominating at pH 6.5 and Zn^{2+}-PCAPH$^-$ at 5.0. Therefore, the observed independence of k_{obsd} on pH in this pH range, as seen in no. 6–9 in Table III, provides strong evidence that the rate constants for Zn^{2+}-$PCAP^{2-}$ and Zn^{2+}-PCAPH$^-$ hydrolysis are very similar. The solvent isotope effect in this pH region is minimal (no. 10–13, Table III), $k_{H_2O}/k_{D_2O} \approx 1.1$. The upturn in rate at pD equal to 7.0 is real, but we were unable to do a good study on rates in the alkaline region because of precipitation problems. Below pH 5, hydrolysis rate studies were limited by side reactions of the pyridine ring. An Arrhenius plot of the data in no. 6, 14, and 15 of Table III gave a $\Delta H^{\ddagger}$ for Zn^{2+}-PCAP hydrolysis of 20 ± 2 kcal/mol and a $\Delta S^{\ddagger}$ value of -21 ± 5 eu. The change in observed rate constant from one plateau level at low PCAP concentration to a higher plateau level at high PCAP concentration (no. 16–21, Table III) provides evidence that Zn^{2+}-PCAP aggregates in solution, perhaps to a dimer, and that the aggregated form is more reactive than the simple 1:1 complex. Ni^{2+}-PCAP hydrolyzes more rapidly than Zn^{2+}-PCAP (no. 22, Table III). In experiments not listed, Zn^{2+}-PCAP hydrolysis was shown to be independent of succinate concentration.

Nucleophilic Catalysis of Zn^{2+}-PCAP Hydrolysis. Added pyridine (up to 0.1 M) has very little effect on the observed rate of phosphate release from PCAP, but causes a marked increase in the rate of Zn^{2+}-PCAP hydrolysis. In Figure 2 the observed rate constant is plotted as a function of pyridine and nicotinamide concentration, giving a biphasic (see inset) curve. Similar results were found with the other substituted pyridines examined in this work. A possible explanation for these results is that pyridine forms a ternary complex with Zn^{2+}-PCAP, which is then subject to nucleophilic catalysis attack by pyridine as shown in Scheme I, where the rate constant for nucleophilic attack on the ternary complex (k_{2t}) is smaller than

Table III. k_{obsd} for PCAP Hydrolysis[b]

No.	PCAP, mM	M^{2+}, M	pH	Temp. °C	No. of runs	Assay method	$k_{obsd} \times 10^3$, min^{-1}
1	0.2		6.0	80	5	P	1.8
2	0.2	Zn^{2+}, 0.003	6.0	80	2	P	2.6
3	0.2	Zn^{2+}, 0.005	6.0	80	2	P	3.0
4	0.2	Zn^{2+}, 0.01	6.0	80	3	P	3.9
5	0.2	Zn^{2+}, 0.02	6.0	80	4	UV, P	4.3
6	0.2	Zn^{2+}, 0.05	6.0	80	10	UV, P	4.8
7	0.2	Zn^{2+}, 0.05	5.0	80	2	UV, P	4.7
8	0.2	Zn^{2+}, 0.05	5.5	80	2	UV, P	4.5
9	0.2	Zn^{2+}, 0.05	6.5	80	2	UV, P	4.6
10	0.2	Zn^{2+}, 0.05	5.5[a]	80	2	UV	4.2
11	0.2	Zn^{2+}, 0.05	6.0[a]	80	2	UV	4.2
12	0.2	Zn^{2+}, 0.05	6.5[a]	80	2	UV	4.6
13	0.2	Zn^{2+}, 0.05	7.0[a]	80	2	UV	6.6
14	0.2	Zn^{2+}, 0.05	6.0	70	2	UV, P	1.8
15	0.2	Zn^{2+}, 0.05	6.0	88	4	UV, P	7.8
16	0.05	Zn^{2+}, 0.05	6.0	80	2	UV	5.0
17	0.1	Zn^{2+}, 0.05	6.0	80	2	UV	5.2
18	0.5	Zn^{2+}, 0.05	6.0	80	2	UV	5.2
19	1.0	Zn^{2+}, 0.05	6.0	80	2	UV	7.4
20	5.0	Zn^{2+}, 0.05	6.0	80	2	UV	11.8
21	10.0	Zn^{2+}, 0.05	6.0	80	2	UV	11.6
22	0.2	Ni^{2+}, 0.05	6.0	80	2	UV	22

[a] pD = pH(read) + 0.4.[29] [b] μ = 2.0.

Scheme I

$$Zn^{2+}\text{-PCAP} + pyr \overset{K_A}{\rightleftharpoons} pyr\text{-}Zn^{2+}\text{-PCAP}$$

$$Zn^{2+}\text{-PCAP} \xrightarrow{k_{2b}[pyr]} Zn^{2+} - PCA + P_i$$

$$Pyr\text{-}Zn^{2+}\text{-PCAP} \xrightarrow{k_{2t}[pyr]} pyr\text{-}Zn^{2+}\text{-PCA} + P_i^{2+}$$

that for attack on the binary complex (k_{2b}). Attempts to demonstrate complex formation by difference spectroscopy in solutions of pyridine and Zn^{2+}-PCAP gave negative results. However, as noted above, there is evidence for self-aggregation of Zn^{2+}-PCAP at concentrations in the millimolar range and it is conceivable that pyridine will bind to Zn^{2+}-PCAP in an analogous way. In this context it is noteworthy that if the rate data are fitted to Scheme I, K_A values of $1\text{-}4 \times 10^3$ M^{-1} are obtained for the substituted pyridines used in this work.

Kirby and Jencks[11] also found biphasic kinetics in their study of nucleophilic attack on *p*-nitrophenyl phosphate and attributed it to self-association of pyridine in solution. However, this explanation is probably not relevant to our findings, since in their study the biphasic nature of the curves first became apparent at much higher pyridine levels (>0.1 M) than in our work.

Catalysis is due to the basic form of the substituted pyridine, as shown first by the fact that the slope of the rate of Zn^{2+}-PCAP hydrolysis plotted against pyridine concentration at two pH values (Figure 3) increases in proportion to the fraction of pyridine present in the basic form and second by the fact that nicotinamide, 4-cyanopyridine, and 3-cyanopyridine are all effective catalysts at pH 6, well above their pK_a values (Table IV). That catalysis by substituted pyridines is nucleophilic rather than general base is suggested by the lack of catalysis by either succinate or 2,6-lutidine[12] (at concentrations up to 0.1 M) and would be in accord with what has been found in several other studies of phosphoryl transfer.[11,13-16] At either 0.05 or 0.1 M pyridine, raising the concentration of Zn^{2+} from 0.05 to 0.1 M had essentially no effect on the observed rate, so that, at least to these concentrations of pyridine, complexing of Zn^{2+} by pyridine is not a problem.

Rate constants evaluated from the final slopes of lines such as those in Figure 2 are listed in Table IV. For pyridine and 4-methylpyridine the slopes measured directly were corrected to slopes for (substituted) pyridine in the basic form. For 3-cyanopyridine, 4-cyanopyridine, and nicotinamide, the correction was unnecessary. A Bronsted plot of these rate constants gives a β value of 0.15 (Figure 4).

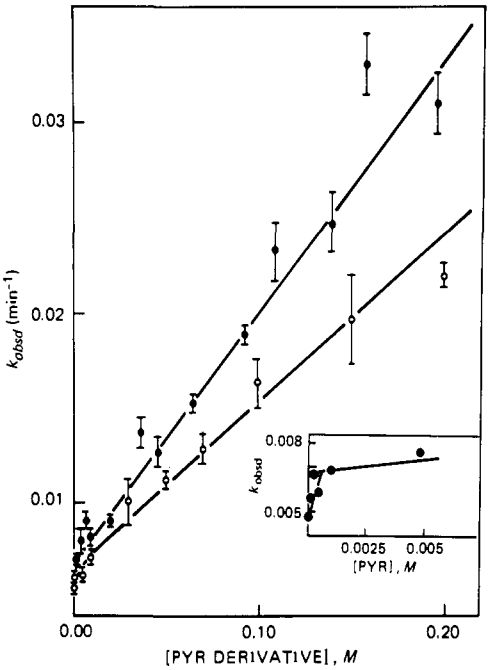

Figure 2. k_{obsd} for PCAP hydrolysis in the presence of saturating Zn^{2+} (0.05 M) and varying concentrations of pyridine [basic form] (●) and nicotinamide (○), pH 6.0, 80 °C, μ = 2 (KCl). Inset: Expanded scale at low pyridine concentration showing biphasic character. Shallow line is the extension of the slope seen in the full figure.

Discussion

In this section we will discuss: (1) the evidence that Zn^{2+}-PCAP undergoes nucleophilic attack by both water and pyridine in the manner of a typical phosphate monoester, with the effect of Zn^{2+} being fully accounted for in terms of leaving-group activation; (2) the intrinsic catalytic effectiveness

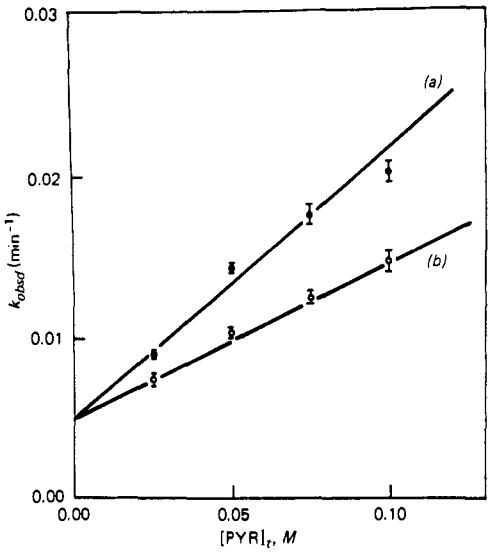

Figure 3. k_{obsd} for PCAP hydrolysis in the presence of saturating Zn^{2+} (0.05 M) and varying concentrations of pyridine at pH 6.5 (●) and pH 5.0 (○), 80 °C, μ = 2 (KCl).

Table IV. Rate Constants for Nucleophilic Attack on Zn^{2+}-PCAP[a]

Nucleophile	pK_a[b]	No. of detm	k, min^{-1} M^{-1}
4-Methylpyridine	5.79	16	0.178
Pyridine	4.85	30	0.135
Nicotinamide	3.35	16	0.078
4-Cyanopyridine	2.22	18	0.116
3-Cyanopyridine	1.46	22	0.026

[a] μ = 2, 80 °C, pH 6.0, 0.2 mM PCAP, 0.05 M Zn^{2+}. In all cases, rate-constant determination by uv difference or phosphate analysis gave the same results. [b] At 80 °C.

of metal ions as leaving-group activators for both hydrolysis and other nucleophilic reactions of phosphate monoesters and the consequences for our own work and related enzymatic reactions; and (3) inferences which can be drawn as to the structure of the transition state for nucleophilic attack on Zn^{2+}-PCAP.

The pK_a of PCAH is 10.0 and that for Zn^{2+}-PCAH is 6.0,[1] and our studies support the conclusion that the catalytic effect of Zn^{2+} on PCAP hydrolysis can be explained on the basis that Zn^{2+}-PCAP hydrolyzes as a typical phosphate monoester with a leaving group of pK_a 6. The evidence is threefold. First, Kirby and Varvoglis[17] found that, at 39 °C, the mono- and dianions of a phosphate monoester with pK_a 5.45 had identical hydrolysis rate constants. Since both leaving-group dependences on pK_a and $\Delta H^{\ddagger}$ are larger for dianion hydrolysis, this pK_a value would be predicted to rise somewhat at 80 °C, so that our finding that Zn^{2+}-PCAPH$^-$ and Zn^{2+}-PCAP^{2-} hydrolyze with similar rate constants is to be expected. Second, the rate constant for Zn^{2+}-PCAP hydrolysis at pH 6 and 80 °C (4.8×10^{-3} min^{-1}) corresponds exactly to that expected for a typical phosphate monoester dianion with a leaving group of pK_a 6 (4.5×10^{-3} min^{-1}), as calculated from the data of Kirby and Varvoglis[17] ($k_{39°C} = 3.2 \times 10^{-5}$ min^{-1}, $\Delta H^{\ddagger}$ = 25.7 kcal/mol). Third, Zn^{2+}-PCAP hydrolysis has a negligible solvent kinetic isotope effect typical of phosphate monoesters,[11,17] but different from what is found for diesters (k_{H_2O}/k_{D_2O} = 1.5)[18] and triesters (k_{H_2O}/k_{D_2O} = 2.0).[19] Similarly, the β value of 0.15 found for substituted-pyridine attack on Zn^{2+}-PCAP is essentially the same as the value of 0.13 found for the phosphate monester *p*-nitrophenyl phosphate (leaving

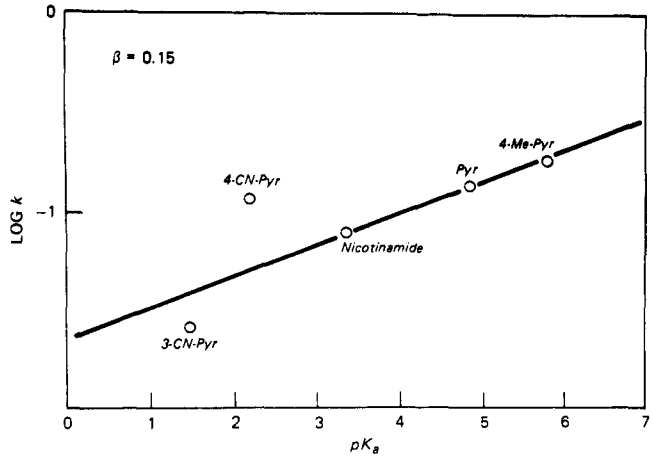

Figure 4. Bronsted plot of log k for nucleophilic attack on Zn^{2+}-PCAP (Table IV) vs. the pK_a of the attacking substituted pyridine.

group pK_a 7.2)[11] and quite different from the value of 0.3–0.4 found for attack on phosphate diesters,[20] and the ratio of the rate constant for pyridine attack on Zn^{2+}-PCAP divided by the rate constant for Zn^{2+}-PCAP hydrolysis (28 M^{-1}) is similar to the corresponding ratio for *p*-nitrophenyl phosphate (16 M^{-1}).[1] A finding which is apparently inconsistent with the picture of Zn^{2+}-PCAP as an ordinary phosphate monoester is the $\Delta S^{\ddagger}$ value for hydrolysis of −21 eu, which is closer to what has been found for hydrolysis of diesters (e.g., $\Delta S^{\ddagger}$ for bis(2,4-dinitrophenyl) phosphate is −25.5 eu),[18] reactions which are thought to have considerable SN2(P) character, than to what has been found for hydrolysis of monoesters ($\Delta S^{\ddagger}$ in the range −5 to +5 eu),[11,13,17,21] reactions which proceed essentially via a "metaphosphate" or SN1(P) mechanism.[22] However, $\Delta S^{\ddagger}$ is particularly unreliable as a diagnostic tool for comparing the hydrolysis of ordinary phosphate monoesters and Zn^{2+}-PCAP, since in the latter case it could include terms reflecting differential solvation of reactant and transition states which do not have to be considered for hydrolyses conducted in the absence of metal ion.

Kirby and Varvoglis[17] measured a Bronsted leaving-group β of −1.23 for phosphate monoester dianion hydrolysis and −0.27 for phosphate monoester monoanion hydrolysis. These results, coupled with the result cited above that, at 39 °C, phosphate monoesters with a leaving group of pK_a 5.45 have equal rates of mono- and dianion hydrolysis, lead to the prediction that, assuming a pK_a of 6.5 for monoanion dissociation to dianion, the observed rate constant for hydrolysis at pH 7 will reflect equal contributions from monoanion and dianion hydrolysis for phosphate monoesters with leaving groups of pK_a 6.0.[31] The hydrolysis at pH 7 of phosphate monoesters with higher leaving-group pK_a's will be dominated by monoanion hydrolysis and those with lower leaving-group pK_a's by dianion hydrolysis. From this prediction it is clear that metal-ion catalysis of phosphate monoester hydrolysis exclusively via a lowering of the leaving-group pK_a will only lead to large ($>10^2$) rate enhancements at pH 7 when the pK_a of the metal-ion bound leaving group is below 5.5. As long as the pK_a remains above this value, even large drops in pK_a on metal-ion binding should lead to only modest rate enhancements. Thus, for a typical phosphate ester with a leaving-group pK_a of 12, even a drop on metal-ion coordination of five units to pK_a 7 would lead to a rate enhancement of $10^{5\times0.27}$, or 22, fold. Maximum pK_a lowering should result from direct coordination of the metal ion to the leaving group and as a model for estimating the magnitude of such an effect one can compare the first hydrolysis constant for the hydrated divalent metal ion to the pK_a for water. In Table V such values are listed for a series of divalent metal ions. The third column of this table lists

Table V. Metal-Ion Hydrolysis Constants (K_H) and Maximum Leaving-Group pK_a for Rate Acceleration of 10^2

	pK_H[a]	pK_W − pK_H[b]	Max leaving-group pK_a[c]
Mg^{2+}	12.8	2.9	7.5
Mn^{2+}	10.6	5.1	10.5
Co^{2+}	8.9	6.8	12.3
Ni^{2+}	9.4	6.3	13.1
Zn^{2+}	8.7	7.0	13.5
Cu^{2+}	6.8	8.9	*d*

[a] Data taken from ref 23. [b] pK_W = 15.7. [c] See text. [d] Any leaving-group pK_a allows rate acceleration by $Cu^{2+} > 10^2$. [e] For 10^2 rate acceleration.[c]

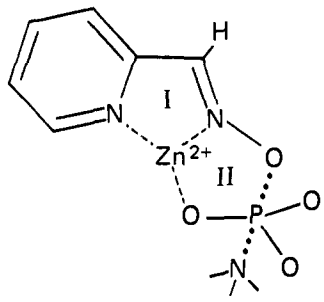

Figure 5. A model for the transition state for amine attack on Zn^{2+}-PCAP and Zn^{2+}-PCA anion attack on phosphoramidates.

the maximum pK_a a leaving group can have in the absence of the metal ion in order that a rate acceleration of 10^2 is obtained on direct coordination of the metal ion to the leaving group. From this table it is clear that for a typical phosphate ester with a leaving group p$K_a > 12$, leaving-group activation by ions such as Mg^{2+} or Mn^{2+} should be rather ineffective in leading to large rate accelerations, activation by ions such as Ni^{2+}, Co^{2+}, and Zn^{2+} should be effective only in those cases where the metal ion can exert a maximal effect on leaving-group pK_a, and only with Cu^{2+} would large rate accelerations be expected as a general result. These considerations thus provide a cogent rationale for the common finding in studies on metal-ion catalysis of phosphate ester hydrolysis that only Cu^{2+} is an effective catalyst.[24] In contrast to hydrolysis, the Bronsted leaving-group β for nucleophilic attack by pyridine is virtually the same for phosphate monoester monoanions (−1.04) as it is for dianions (−1.03),[14] so that large catalytic effects on the rate of pyridine attack should be observed from even rather modest drops in leaving-group pK_a over a wide pH range. Thus, direct leaving-group coordination by even as weakly acidic a metal ion as Mg^{2+} could lead to a rate enhancement of 10^3.

The above discussion provides the basis for understanding our own results. Assuming that PCAP is a typical phosphate monoester with a leaving-group pK_a of 10 allows calculation, again from the data of Kirby and Varvoglis,[17] of a hydrolysis rate at 80 °C of 2.5×10^{-3} min^{-1} for $PCAPH^-$ and 3.1×10^{-8} min^{-1} for $PCAP^{2-}$. Since $PCAPH^-$ has a pK_a of 5.8 for dissociation to $PCAP^{2-}$ (Table I), these rate constants lead to a predicted k_{obsd} at pH 6 of 1.0×10^{-3} min^{-1}, which is essentially identical with the upper-limit rate constant for eq 2 of 0.85×10^{-3} min^{-1} (see Results). Assuming this upper-limit estimate to be correct, we see that despite the fact the rate constant for Zn^{2+}-$PCAP^{2-}$ hydrolysis is 1.5×10^5 times faster than the calculated rate constant for $PCAP^{2-}$, the observed catalytic effect of Zn^{2+} at pH 6, as measured by the ratio of the rate constants for eq 1 and 2, is only fivefold. In terms of our general discussion, the pK_a lowering of four units resulting from indirect coordination of Zn^{2+} to the leaving group is insufficient to result in a large observed catalysis. On the other hand, a pK_a lowering of four units should yield a 10^4-fold rate enhancement for pyridine attack, and this explains our finding that pyridine catalyzes Zn^{2+}-PCAP hydrolysis, while having little effect on PCAP hydrolysis.

Enzymes catalyzing phosphoryl transfer, such as hydrolases or kinases, often require divalent metal ions for activity, with Mg^{2+} or Mn^{2+} usually conferring the highest activity.[25,26] As we have seen, these ions could be effective as leaving-group activators in reactions involving nucleophilic attack, as in kinases, but not for direct hydrolysis reactions. One way a hydrolase could use Mg^{2+} as a leaving-group activator would be if the actual attacking nucleophile were hydroxide ion rather than water. The results of a solvent isotope effect study on the enzyme yeast inorganic pyrophosphatase[27] are consistent with such a mechanism.

Nucleophilic attack by pyridines on Zn^{2+}-PCAP is almost the exact microscopic reverse of Zn^{2+}-PCA attack on phosphoramidates, as studied in the accompanying paper,[2] differing only in that alkyl and aralkyl amines rather than substituted pyridines were used. However, this difference in the nature of the amine used in opposite directions of reaction should have little effect on the interpretation of our results, since Benkovic and Sampson[28] have shown that leaving-group β values for phosphoramidate hydrolysis were similar for both substituted pyridines and alkyl and aralkyl amines. The low nucleophilic β (0.15) for substituted pyridine attack on Zn^{2+}-PCAP and the large negative leaving β (−0.8) for Zn^{2+}-PCA attack on phosphoramidates support the notion that both reactions proceed via a common transition state in which there is small bond order between the phosphorus and either the incoming or leaving amine and is thus, in this respect, little different from the transition state for phosphoryl transfer in the absence of metal ions.[22a] We picture this transition state as shown in Figure 5, in which the two nitrogens of PCA and the phosphoryl group form a tridentate ligand for Zn^{2+}. We previously have explained the large catalytic effect of Zn^{2+} on PCA anion attack at phosphoramidates in terms of its roles as a template and in reducing the repulsive charge-charge interaction between the PCA anion and the phosphoryl dianion center[1,2] and the large catalytic effect of Zn^{2+} on pyridine attack on PCAP in terms of its role as a leaving-group activator. An alternative explanation for catalysis in both directions of the reaction could be made on the basis of a more favorable interaction of Zn^{2+} with the transition state than with either of the reactant states. This is clearly the case for the reactant state for Zn^{2+}-PCA attack on phosphoramidates, in which chelate ring II, present in the transition state (Figure 5), is lacking. Chelate ring II may be present in the reactant state for pyridine attack on Zn^{2+}-PCAP (see Results), although it clearly does not contribute much to the strength of PCAP interaction with Zn^{2+}, since the Zn^{2+} binding constant to $PCAP^{2-}$ (790 M^{-1}, Table I) is only a factor of five higher than the Zn^{2+} binding constant to PCAH (150 M^{-1}). There are two reasons for expecting a stronger interaction of Zn^{2+} with the transition state than with $PCAP^{2-}$. First, if, by analogy with all other known phosphate monoester reactions, the P-O bond is largely broken in the transition state,[32] then the PCA moiety should resemble the PCA anion, which has a much stronger interaction with Zn^{2+} than PCAH, as shown by its binding constant of 10^6 M^{-1}.[1] Second, expansion of ring II in the transition state as a result of P-O bond cleavage could result in a decrease in ring strain arising from the formation of two fused five-membered rings, and thus to a stronger net interaction of Zn^{2+} with phosphate.

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(31) Assuming that the monoanion leaving-group β is also -0.27 at 39 °C.
(32) In support of this hypothesis, one can calculate a Bronsted leaving-group β of -1.2 for M^{2+}–PCAP hydrolysis from the rate constants for Zn^{2+}–PCAP and Ni^{2+}–PCAP hydrolysis (Table III) and the pK_a values for Zn^{2+}–PCAH (6.0) and Ni^{2+}–PCAH (5.45).

Metal-Ion Catalysis of Phosphoryl Transfer via a Ternary Complex. Effects of Changes in Leaving Group, Metal Ion, and Attacking Nucleophile

Chih-Min Hsu and Barry S. Cooperman*

Contribution from the Department of Chemistry, University of Pennsylvania, Philadelphia, Pennsylvania 19174.

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Abstract: Our earlier work[2a] showing rapid phosphoryl transfer in the ternary complex pyridine-2-carbaldoxime–Zn^{2+}–phosphorylimidazole is here extended by studies of the effects of (a) substituting Ni^{2+} for Zn^{2+}, (b) substituting a series of phosphoramidates for phosphorylimidazole, and (c) substituting pyridine-2-aldehyde hydrazone for pyridine-2-carbaldoxime. The results with Ni^{2+} are qualitatively similar to those obtained previously with Zn^{2+} and allowed a clearer demonstration of the need for a protonated imidazole ring in the reactive ternary complex. The rates of transfer for a series of phosphoramidates permit calculation of a Bronsted leaving-group β which is similar to that obtained for simple phosphoramidate hydrolysis. No transfer to the hydrazone is detected. These results are interpreted as showing that the catalysis of phosphoryl transfer by Zn^{2+} results from both charge-shielding and template effects and that Zn^{2+} has little direct effect on the phosphoryl group itself.

Introduction

Phosphoryl-transfer reactions are among the most common in biological systems. The enzymes catalyzing these reactions generally require divalent metal ions for activity. In the last decade a great deal of effort has been directed toward understanding the mechanism of phosphoryl transfer[1] and elucidating the possible catalytic roles of metal ions in the reaction.[2] Several recent reviews have appeared summarizing work in this area.[1,3]

A previous publication from this laboratory reported on the Zn^{2+}-requiring phosphoryl transfer from phosphorylimidazole (PIm) to pyridine-2-carbaldoxime (PCA) anion and provided evidence that this transfer proceeded via the ternary complex PCA–Zn^{2+}–PIm.[2a] The importance of this reaction was that it represented a clear example of anionic oxygen attack on a phosphoryl dianion, a reaction which though common enzymatically had been difficult to demonstrate in model systems. This paper extends our earlier work by measuring the effect of varying the leaving-group amine on the rate of phosphoryl transfer. In addition, the effects of substituting Ni^{2+} for Zn^{2+} and pyridine-2-aldehyde hydrazone (PAH) for PCA have also been determined.

Experimental Section

Materials. Calcium phosphorylimidazole (PIm),[2a] *N*-methyl phosphorylimidazole (*N*-MePIm),[2a] potassium ammonium phosphoramidate (PA),[4,5] potassium *N*-(phenethyl)phosphoramidate (*N*-(phenethyl)PA),[6] potassium *N*-(benzyl)phosphoramidate[6] (*N*-(benzyl)PA), and pyridine-2-aldehyde hydrazone (PAH)[7] were prepared as previously described. *N*-(*n*-Butyl)phosphoramidate (*N*-(*n*-butyl)PA) was a gift of Professor S. J. Benkovic. Pyridine-2-carbaldoxime (PCA) and *N,N'*-dimethylpiperazine were obtained from Aldrich and the latter was redistilled before use. $ZnSO_4 \cdot 7H_2O$, $NiCl_2 \cdot 6H_2O$, and KCl were Baker Analyzed Reagents. 2-[*N*-Morpholino]ethanesulfonic acid (Mes) was obtained from Sigma.

Kinetic Procedures. Rates of phosphoryl transfer from phosphoramidates to Zn^{2+}–PCA and Ni^{2+}–PCA were measured by a modification of the modified Martin–Doty procedure of Jencks and Gilchrist, as described previously.[2a] Reaction mixtures were generally 0.6 mM in the phosphoramidate under study and were brought to an ionic strength of 0.5 with KCl. Temperature variation was within ±0.1°C. The pH of reaction mixtures was measured before and after the kinetic runs and never varied by more than ±0.1 pH unit. All kinetic runs obeyed first-order kinetics. Reactions were generally followed for 2 half-lives and at least six points were taken per run. All rate constants were measured at least twice. Reproducibility was ±5%. Measurements of pH at 10 °C were by a standard procedure.[8]