Biomimetically Activated Amino Acids. Catalysis in the Hydrolysis of Alanyl Ethyl Phosphate

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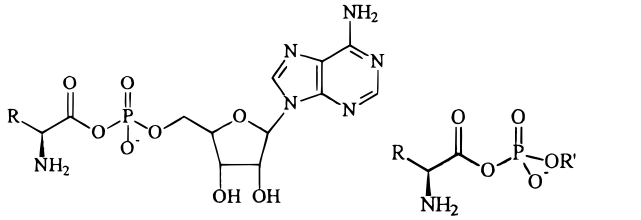
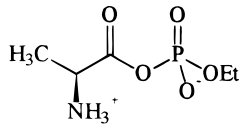
Abstract: Alanyl ethyl phosphate (**1**) is an activated derivative of alanine that is functionally related to the corresponding aminoacyl adenylate, the initial activated amino acid intermediate in protein biosynthesis. To establish the inherent reactivity of these species, the kinetic parameters for hydrolysis of alanyl ethyl phosphate in water at 25 °C were determined. There is catalysis by acid ($k = 4 \times 10^{-4}$ $M^{-1}s^{-1}$) and base ($k = 1.7$ M^{-1} s^{-1}) along with two pH-independent processes ($k = 3 \times 10^{-5}$ and 1.6×10^{-3} s^{-1}) that are connected as a kinetic titration curve of the amino group of alanyl ethyl phosphate ($pK_a = 7.8$). The results are consistent with mechanisms proceeding via addition to the carbonyl of water or hydroxide with proton migrations. Reaction with methanol is slower than reactions with water while reaction with 2-propanol leads to complex products. In solutions sufficiently concentrated for ^{31}P NMR analysis, alanyl ethyl phosphate also undergoes reactions that produce alanylalanine and other condensation products. Metal ions catalyze the hydrolysis reactions through complex formation. Cupric and zinc ions are most effective (∼100-fold larger rate constant than water: association constants > 100 M^{-1}) with magnesium and calcium forming weaker and less reactive complexes. These results show that aminoacyl alkyl phosphates are sufficiently stable to be used in water and that metal ions can facilitate their reactions. Improved catalysts will be needed to facilitate biomimetic processes such as aminoacylation of t-RNA.

The formation of proteins requires activation of the carboxyl group of each amino acid that is added. In most cells, activation results from the reaction of the amino acid and ATP to form a mixed anhydride, an aminoacyl adenylate, as an enzyme-bound intermediate. The aminoacyl adenylate goes on to acylate the terminal 3′-hydroxyl group of the cognate t-RNA.[1,2] The mixed anhydride is a member of the general class of aminoacyl phosphate monoesters[3] and is one of the principal processes of molecular activation in biochemistry.[4] Although the reaction patterns of aminoacyl adenylates are significant in catalysis of this key biological process,[5] little is known about these compounds since they are not readily available.[6] How reactive are they? How can their reactivity be enhanced and perhaps put to use? The inherent reaction patterns should arise from the aminoacyl phosphate ester function, independent of the adenosine moiety. Since we recently developed a convenient preparation of aminoacyl phosphate esters,[3] we have been able to produce pure compounds in large quantities and begin systematic studies.



We noted that the compounds are relatively stable in water but that they can react rapidly with amine nucleophiles.[3] In this paper we focus on the mechanisms and catalysis of hydrolysis of alanyl ethyl phosphate (**1**) as a typical member of the general class. Aminoacyl alkyl phosphates may also be useful for reactions that mimic those of aminoacyl adenylates with t-RNAs, transferring the aminoacyl moiety to a terminal ribose hydroxyl. Thus, we have also assessed the reactivity of **1** with methanol and 2-propanol as preliminary models. We find that these reactions are slow and complex, establishing the need for the development of catalysts for aminoacyl transfer to weak nucleophiles.



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Experimental Section

Methods. Commercial reagents were used as received. NMR spectra were recorded at 200, 300, or 400 MHz for ^1H, 100 MHz for ^{13}C, and 121 or 160 for ^{31}P. Kinetic runs were carried out in a jacketed reaction vessel with a thermoregulated recirculating water bath.

Kinetics. Since alanyl ethyl phosphate is an anhydride of a carboxylic acid and the monobasic form of ethyl phosphate, a significant amount of acid is released during hydrolysis in neutral and alkaline solutions. Therefore, a "pH-stat" was used to measure rates of appearance of products at pH 7 and higher. The jacketed reaction flask contained a magnetic stirrer, a glass electrode, and an input tube from an automatic buret containing 0.01 M sodium hydroxide solution. The system was continuously flushed with nitrogen (to minimize absorption of carbon dioxide). A solution of alanyl ethyl phosphate (0.5 mL of 0.005 M) was transferred into the reaction vessel and sufficient 1.0 M potassium chloride was added to bring the total volume to 5 mL. The volume of base added from the buret was recorded as a function of time (through a computer), for approximately 4 half-lives. The data

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(3) Kluger, R.; Li, X.; Loo, R. W. *Can. J. Chem.* **1996**, *74*, 2395−400.
(4) Metzler, D. *Biochemistry, the Chemical Reactions of Living Cells*; Academic Press: New York, 1977; Chapter 11.
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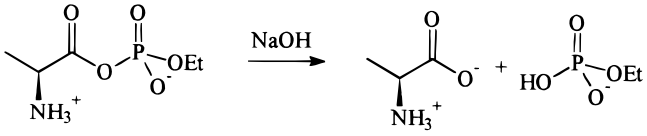
Scheme 1



were fit to the integrated first-order rate law by nonlinear regression. The pseudo-first-order kinetic data were used to measure dependence on concentrations of added species.

For reactions in solutions more acidic than pH 7, the conversion of reactant to product was followed by periodic recording of ^{31}P NMR (121 MHz). The integrals of the signal corresponding to alanyl ethyl phosphate were noted relative to that of a standard solution of disodium pyrophosphate (0.1 M in deuterium oxide) in a sealed capillary tube (also serving as a "lock signal" for the NMR). For buffer dependence studies, 1.0 mL of 0.025 M alanyl ethyl phosphate in sodium acetate buffer concentrations of 0.2 to 1.0 M (I = 1.0 with sodium chloride as needed) was placed in 5-mm NMR tubes. Spectra were obtained with 128 scans and a 2-s delay in the broad band proton-decoupled mode. Data collection for each spectrum took approximately 4 min. Spectra were taken at varying intervals for 4 to 5 half-lives. Between data collections, samples were kept at 25 °C in a circulating water bath. Acidic solutions were prepared from titrated 1 M HCl that was diluted with 1 M sodium chloride solution. Reactions were followed as described above.

Data analysis and fitting was done by nonlinear regression on a computer. For the ^{31}P NMR data, the relative values of the integrals of the signals corresponding to alanyl ethyl phosphate and disodium pyrophosphate were fitted to a first-order exponential decay. This gave an observed first-order rate coefficient for the disappearance of alanyl ethyl phosphate. For the pH-stat data, the volumes of added sodium hydroxide solution were fitted to a first-order growth curve to obtain an observed first-order rate coefficient for the disappearance of alanyl ethyl phosphate.

Synthesis. Tetra-*n*-butylammonio-*N*-t-boc-(L)-alanyl ethyl phosphate was prepared according to the reported procedure[3] by using dicyclohexyl carbodiimide (0.0011 mol) to couple *N*-(t-boc)-(L)-alanine (0.0010 mol) and bis(tetra-*n*-butylammonium)ethyl phosphate (0.0011 mol) in dichloromethane (20 mL). The product is a colorless oil: (76% yield) ^{1}H NMR (200 MHz, chloroform-*d*) δ 1.19 (t, 3H), 1.30 (t, 12H), 1.34 (d, 3H), 1.38 (s, 9H), 3.36 (q, 8H), 3.97 (m, 2H), 4.21 (m, 1H), 5.15 (d, 1H); ^{31}P NMR (chloroform-*d*) δ −6.8.

(L)-Alanyl ethyl phosphate was prepared by treatment of tetraethylammonium-*N*-t-boc-(L)-alanyl ethyl phosphate with trifluoroacetic acid. Overall yield: 40% from *N*-(*tert*-butoxycarbonyl)-(L)-alanine): mp 120 °C dec; IR (KBr) 1764, 1624, 1574, 1251, 1230, 1075, 1047 cm^{-1}; ^{1}H NMR (200 MHz, deuterium oxide) δ 1.19 (t, 3H), 1.50 (d, 3H), 3.95 (m, 2H), 4.16 (q, 1H); ^{13}C NMR (100 MHz, deuterium oxide) δ 15.63 (s), 16.22 (d, J^{C-P} = 6.6 Hz), 49.95 (d, J^{C-P} = 7.3 Hz), 64.78 (d, J^{C-P} = 6.6 Hz), 167.92 (d, J^{C-P} = 8.8 Hz); ^{31}P NMR (121 MHz, deuterium oxide) δ −6.58. FAB MS calculated 197, found (*m*/*z*) 196 (M − H). All further discussion of this compound omits the stereochemical descriptor.

Results

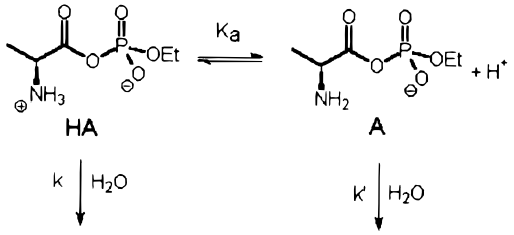
The hydrolysis of alanyl ethyl phosphate produces alanine and ethyl phosphate (Scheme 1).

Both the zwitterionic (HA) and anionic forms (A) of alanyl ethyl phosphate should undergo hydrolysis (Scheme 2) as given by the rate law in eq 1. S_T is the concentration of alanyl ethyl phosphate in all forms; f_{HA} and f_A are the fractions in the zwitterionic form and anionic forms, respectively.

$$k_{obs}[S_T] = (kf_{HA} + k'f_A)[S_T] \quad (1)$$

A series of observed first-order rate coefficients was measured by pH-stat in solutions whose pH brackets the pK_a of the zwitterionic form of alanyl ethyl phosphate (see below). A plot

Scheme 2



of k_{obs} versus pH gives a curve that fits a titration equation, with the apparent pK_a from kinetics being identical with the value found by equilibrium titration (=7.8 ± 0.1). The kinetic and thermodynamic titration curves are shown in Figure 1.

The data in Figure 1A were fit to a titration curve equation to give as limits the rate constants for the uncatalyzed hydrolysis of the zwitterionic form (below pH 6) and the anionic form (above pH 9). The lower rate was determined independently by extrapolation of rates of reactions in buffered solutions (see below). These values are also determined by fitting the data to a plot of rate coefficient versus fraction of all material in the anionic form.[7] The rate constant obtained from the plot for hydrolysis of the zwitterionic form of alanyl ethyl phosphate is (3.9 ± 0.4) × 10^{-5} s^{-1}. A plot of observed first-order rate coefficient versus free base fraction (8 points: 0.2 to 0.8) extrapolated to unity gives k_{OH} = (1.6 ± 0.2) × 10^{-3} s^{-1}.

Base Catalysis. In more alkaline solutions (reaction measured for six points between pH 10 and 11), the rate of hydrolysis is proportional to hydroxide concentration. This is likely to involve the reaction of hydroxide with the anionic form of the substrate (**A** in Scheme 2). The slope of the plot of observed first-order rate coefficients versus hydroxide concentration gives the second-order rate constant for specific base catalyzed hydrolysis of alanyl ethyl phosphate: (1.67 ± 0.04) M^{-1} s^{-1}.

Water Catalysis and Acid Catalysis. There is not a sufficient release of proton equivalents generated by the hydrolysis of alanyl ethyl phosphate below pH 7 to permit the reaction to be followed by a pH-stat in dilute solutions. For these cases ^{31}P NMR was used to follow the consumption of reactant (by the integrated signal from alanyl ethyl phosphate versus an external standard). The first-order rate coefficient for hydrolysis of alanyl ethyl phosphate in weakly acidic solutions was obtained for a series of acetate/acetic acid buffers (0.2, 0.4, 0.6, 0.8 M; see Experimental Section for details) between pH 4.0 and 5.0. The buffers catalyze the reaction (first order in buffer concentration). The first-order rate constant for the reaction in the absence of buffer is obtained by extrapolation to [buffer] = 0: pH 4.0, k = (3.2 ± 0.3) × 10^{-5} s^{-1}; pH 4.5, k = (3.4 ± 0.3) × 10^{-5} s^{-1}; pH 5.0, k = (3.4 ± 0.4) × 10^{-5} s^{-1}. The combination gives a pH-independent rate constant of (3.3 ± 0.6) × 10^{-5} s^{-1} for the spontaneous hydrolysis pathway of the zwitterionic form.

The hydrolysis of alanyl ethyl phosphate is acid catalyzed: (0.1 M HCl, k_{obs} = 4.8 × 10^{-5} s^{-1}; 0.5 M HCl, k_{obs} = 2.3 × 10^{-4} s^{-1}; 1.0 M HCl, k_{obs} = 3.8 × 10^{-4} s^{-1}). The second-order rate constant for specific acid catalysis is (3.8 ± 0.5) × 10^{-4} M^{-1} s^{-1} from the linear correlation of the observed first-order rate coefficients and acid concentrations.

Overall Rate Law. A rate law from four mechanisms that predominate in different conditions is summarized in eq 2. This

(7) Jencks, W. P. *Catalysis in Chemistry and Enzymology*; McGraw-Hill: New York, 1969; Chapter 11.

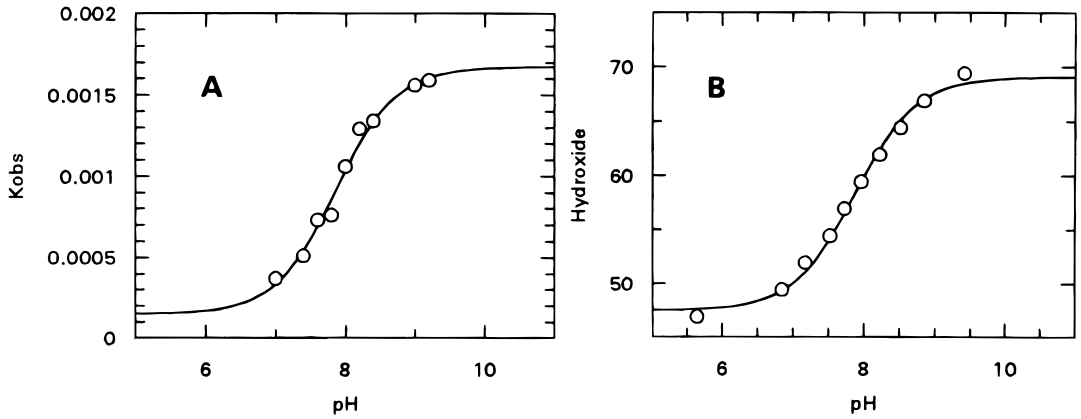


Figure 1. (A) pH vs rate coefficient (s^{-1}) for the hydrolysis of alanyl ethyl phosphate. (B) Titration of alanyl ethyl phosphate with sodium hydroxide (relative volume). Both fit the standard titration equation with the pK_a for the amino group of alanyl ethyl phosphate as 7.8 ± 0.1.

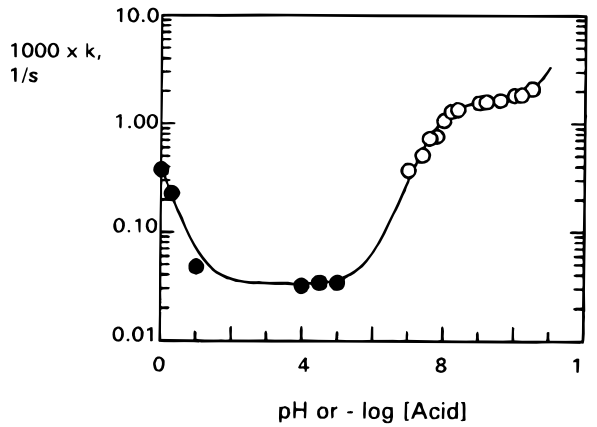


Figure 2. pH vs rate coefficient profile for hydrolysis of alanyl ethyl phosphate (25 °C) as described in the text: (●) data from ^{31}P NMR; (○) data from a pH-stat.

Table 1. Rate Constants for the Hydrolysis of Alanyl Ethyl Phosphate (25 °C) Derived from Fitting Experimental Data to Eq 1

term	value
k^{H}	$(3.8 \pm 0.5) \times 10^{-4}$ M^{-1} s^{-1}
k^{HA}	$(3.3 \pm 0.6) \times 10^{-5}$ s^{-1}
k^{A}	$(1.6 \pm 0.2) \times 10^{-3}$ s^{-1}
k^{OH}	(1.67 ± 0.04) M^{-1} s^{-1}

was used to generate a pH−rate profile (Figure 2) with the rate constants in Table 1.

$$v = k_{obs}[\mathbf{1}] = (k_H[H^+] + k^{HA}f_{HA} + k^{A}f_{A} + k_{OH}[OH^-])[\mathbf{1}] \quad (2)$$

Complications. Since alanyl ethyl phosphate and alanine (from hydrolysis) contain an amino group, they can also react as nucleophiles with the electrophilic carbonyl center of alanyl ethyl phosphate. At higher concentrations of substrate, this competes with hydrolysis since the free amino group is a much better nucleophile than water toward alanyl ethyl phosphate and it is present in reasonable quantities at pH > pK_a of the zwitterionic form. Thin-layer chromatography and ^{1}H NMR indicated the formation of such products. Since these result from reactions that are second order in aminoacyl compound, they are promoted by the high concentrations used for NMR-based kinetic studies. The solutions used for pH-stat kinetics do not lead to significant second-order processes since the samples are dilute. A further complication of the NMR method is the necessary use of buffers. They catalyze the reaction, requiring the rate due to buffer to be separated.

Reaction of Alanyl Ethyl Phosphate with Alcohols. As a preliminary to the use of these materials for aminoacylation of t-RNA, we examined the inherent reactivity of alanyl ethyl phosphate with alcohols. Alanyl ethyl phosphate reacts with methanol to give alanine methyl ester and ethylphosphoric acid. The identity of alanine methyl ester was confirmed by comparison of ^{1}H NMR and thin-layer chromatographic patterns to those for genuine alanine methyl ester. The rate of methanolysis of alanyl ethyl phosphate in methanol, by ^{31}P NMR at 25 °C, is very slow, with an observed first-order rate constant of (6.8 ± 0.2) × 10^{-5} s^{-1} ($t_{1/2}$ = 170 min).

The reaction of alanyl ethyl phosphate in 2-propanol (a model for the 2° alcohol of the ribosyl ring of t-RNA) is even slower. The cloudy suspension of alanyl ethyl phosphate undergoes only a partial reaction over a period of days. After 1 week at room temperature, the ^{31}P NMR indicates 86% of the alanyl ethyl phosphate remained intact. ^{1}H NMR analysis of the crude mixture does not indicate a signal for a 2-propyl group although alanine residues other than alanyl ethyl phosphate are evident.

To increase the rate of the reaction between alanyl ethyl phosphate and 2-propanol, heat and base were applied. (Alanyl ethyl phosphate is soluble in refluxing 2-propanol.) After 24 h, ^{31}P NMR indicated that all the starting material was consumed. Both chromatography and ^{1}H NMR revealed a mixture of products. Similarly, a number of products appeared when the reaction was repeated at room temperature with an equivalent of triethylamine or imidazole. These products were not identified (this is the subject of ongoing studies).

Metal Ion Catalysis. Initially, the effects of the divalent ions of copper, zinc, magnesium, and calcium on the rate of hydrolysis of alanyl ethyl phosphate [0.0005 M] were screened with metal ion concentrations of 0.005 M at pH 7. Cupric and zinc ions cause a significant increase at these low levels, while magnesium and calcium do not. The detailed concentration dependence was then measured.

The effect of variation in ion concentration on the rate of the reaction was found for each metal. The addition of each increased the observed first-order rate coefficient up to saturation. The curves fit the equation for saturating binding. As in other catalytic systems, the fit provides an apparent association constant for the metal and substrate as well as the rate constant for the decomposition of the metal complex. The curve for catalysis by cupric ion is shown in Figure 3.

The dependence of rate on zinc, magnesium, and calcium ions was also found. All enhance the reaction, but the effects of magnesium and calcium ions are smaller. These results are shown in Figures 4, 5, and 6.

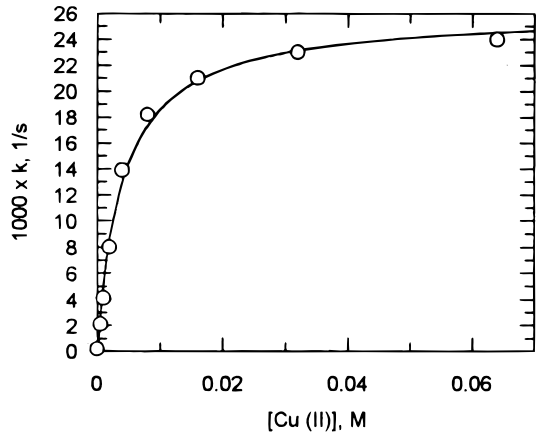


Figure 3. Dependence of observed first-order rate coefficient for hydrolysis alanyl ethyl phosphate.

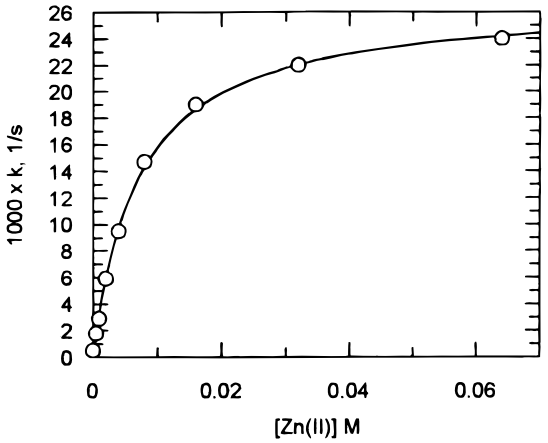


Figure 4. Effect of zinc ion concentration on the observed rate coefficient for hydrolysis of alanyl ethyl phosphate.

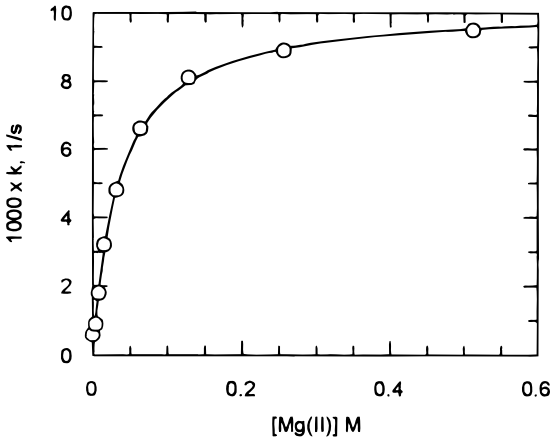


Figure 5. Effect of magnesium ion concentration on the observed first-order rate coefficient for hydrolysis of alanyl ethyl phosphate.

The simplest scheme that can be used to fit the data in each saturation plot is shown in Scheme 3.

The association constant, K_1, and the maximal rate constant, k_2, are determined from the fit of the data to the equation for each plot, where **S** is alanyl ethyl phosphate and **M** represents divalent metal ions.

$$k_{obs} = K_1 k_2[\mathbf{M}]/(1 + K_1[\mathbf{M}]) \quad (3)$$

The rate constants and association constants obtained from fitting to give these plots are given in Table 2.

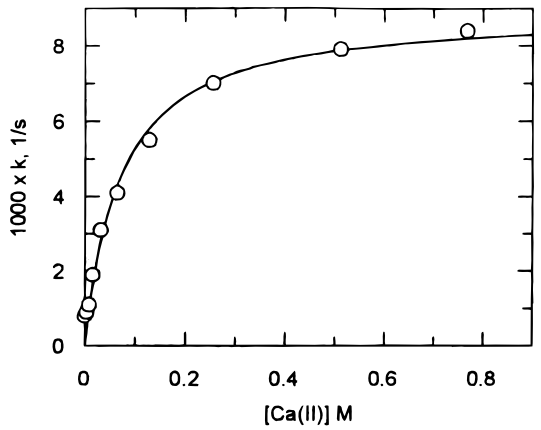


Figure 6. Effect of calcium ion concentration on the observed first-order rate coefficient of hydrolysis of alanyl ethyl phosphate.

Scheme 3

$$S + M \underset{}{\overset{K_1}{\rightleftharpoons}} S.M \xrightarrow{k_2} P + M$$

Table 2. Rate and Association Constants from Fitting Kinetic Data (Scheme 3 and Figures 3−6) for Metal-Catalyzed Hydrolysis of Alanyl Ethyl Phosphate at pH 7.0, 25 °C[a]

metal	k_2, s^{-1}	k_2/k_{obs}	K_1, M^{-1}
Cu(II)	2.6×10^{-2}	100	250
Zn(II)	2.7×10^{-2}	135	141
Mg(II)	1.0×10^{-2}	50	28
Ca(II)	0.9×10^{-2}	45	15

[a] The "k_2/k_{obs}" entry refers to the acceleration observed for the metal complex at pH 7.

Discussion

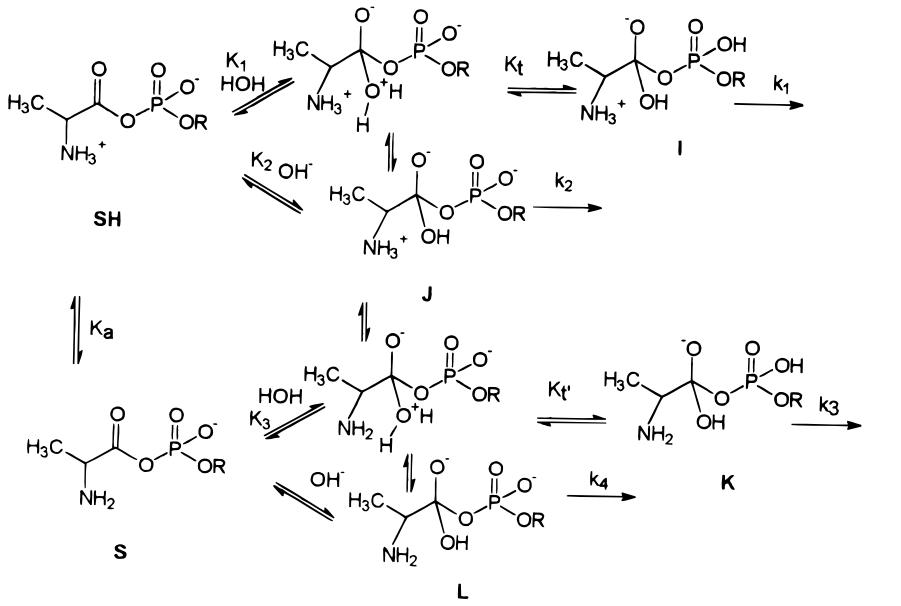
The hydrolysis of alanyl ethyl phosphate in water follows the general reaction patterns of acyl phosphate monoesters,[8−12] complicated by the presence of the α-amino group. Since the amino group exists in protonated and neutral forms, it alters the reactivity at the acyl center. Scheme 4 summarizes a kinetic scheme that can be used to connect the data to reaction mechanisms in neutral and alkaline solutions. The mechanisms are consistent with the data and are distinguished only by protonation states.

Alanyl ethyl phosphate is shown in the zwitterionic (**SH**) and anionic (**S**) forms that are the major species in neutral solutions. The acidity constant K_a connects **S** (plus a proton) and **SH**. A set of tetrahedral intermediates can form by the addition of water or hydroxide ion to **S** and **SH**.

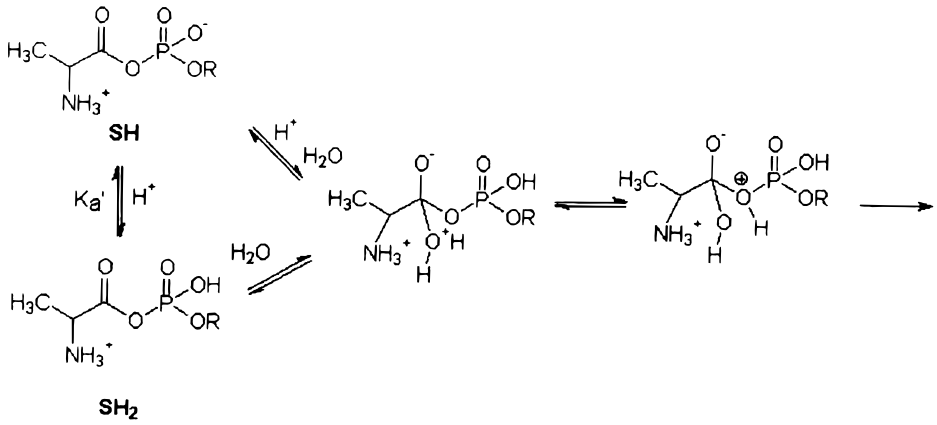
These intermediates in Scheme 4 are tautomeric forms that can revert directly to reactants; they are also likely to lose a proton since the least basic sites are protonated. Loss of the proton from the ether oxygen leads to the same intermediates as formed by addition of hydroxide (**I** and **L**). A shift of the same proton gives the somewhat more stable intermediates, **I** and **K**. These can expel ethyl phosphate as the monoanion rather than as the more basic dianion. (Some of the intermediates differ by the position of a proton and are therefore difficult to distinguish. The addition of hydroxide to **SH** leads to transition states with the same net charge, as does addition of water to **S**.) Addition of hydroxide may be rate determining since its reverse is likely to be slower than the competing loss

(8) Disabato, G.; Jencks, W. P. *J. Am. Chem. Soc.* **1961**, *83*, 4400.
(9) Disabato, G.; Jencks, W. P. *J. Am. Chem. Soc.* **1961**, *83*, 4393.
(10) Kluger, R.; Tsui, W.-C. *J. Org. Chem.* **1980**, *45*, 2723.
(11) Kluger, R.; Grant, A. S.; Bearne, S. L.; Trachsel, M. R. *J. Org. Chem.* **1990**, *55*, 2864−8.
(12) Wodzinska, J. M., Ph.D. Thesis, University of Toronto, 1994.

Scheme 4



Scheme 5



of ethyl phosphate. Where the reaction involves addition of water, its reverse should be faster than loss of ethyl phosphate (in any ionization state), since neutral water is a better leaving group. Proton transfers among the reactive intermediates may also be rate determining since the concentrations of these species are low, even if they are associated with large rate constants.

The pH−rate profile in Figure 2 shows a pH-independent rate in the region that corresponds to the alanyl ethyl phosphate being present as **SH** (Scheme 4). The rate is consistent with the addition of water to **SH**, tautomerization to form **I**, and elimination (k_1). In solutions with pH > pK_a for loss of the proton of the ammonium ion of **SH**, the reaction has a larger water rate. This can involve a route via **J** or **K**. In acyl phosphate esters the rate of reaction has a dependence of the basicity of the attacking group (β_{nuc}) of 0.9,[12] indicating that the transition state for addition resembles the intermediate. The elimination step, as the microscopic reverse, should have a similar transition state. Both the alkyl phosphate monoanion and dianion are accessible in neutral solutions, so neither must be avoided. The lesser extent of protonation of the amino group is exactly compensated by the increase in hydroxide concentration as pH increases. Therefore, the route via **J** should be favored over that via the less prevalent tautomer, **K**. Thus, the rate constant for the water reaction in the upper plateau could also give the second-order rate constant for the hydroxide dependent hydrolysis ($(3.2 \pm 0.3) \times 10^3$ M^{-1} s^{-1}).

The presence of a hydroxide-catalyzed reaction of alanyl ethyl phosphate begins to appear in the high pH range of Figure 2. The most likely route is addition of hydroxide to **S**, forming **L**. Although **S** is somewhat less electrophilic than **SH** because of the relative electron withdrawing ability of the amino and ammonio substituents, the increased hydroxide concentration favors the route via **L** at high pH.

There is also an acid-catalyzed route involving **SH** as demanded by the data in Figure 2 that show an increase in acidic solutions. Scheme 5 suggests the likely pathway: through reasonable proton transfers, the intermediate can expel neutral ethyl phosphoric acid, an excellent leaving group.

The electron-withdrawing effect of a protonated amino group α to the carbonyl of an acyl phosphate should accelerate its rate of hydrolysis ($\sigma_i = 0.60$)[13] compared to that of unsubstituted acyl phosphates. On the other hand, the unprotonated amino group does not enhance the reaction electronically ($\sigma_i = 0.10$).[13] Information in Table 3 shows where the effects of the amino group are most significant. The rate constant for the acid-catalyzed hydrolysis of alanyl ethyl phosphate at 25 °C is somewhat greater than that of benzoyl methyl phosphate at 58 °C.[12] For a typical activation barrier this corresponds to a factor of about 10. The "water reaction" in acidic solution has a larger difference (∼30-fold or ∼300 at the same temperature). The

(13) Charton, M. *J. Org. Chem.* **1964**, *29*, 1222.

Table 3. Rate Constants for the Hydrolysis Alanyl Ethyl Phosphate and Benzoyl Methyl Phosphate

acyl phosphate	conditions	k_H (M^{-1} s^{-1})	k_{H_2O} (s^{-1})	k_{OH} (M^{-1} s^{-1})
benzoyl methyl phosphate	58 °C, $I = 1.0$	1.9×10^{-4}	6.5×10^{-7}	2.4
alanyl ethyl phosphate	25 °C, $I = 1.0$	3.9×10^{-4}	2.0×10^{-5}, 1.6×10^{-3}	1.67

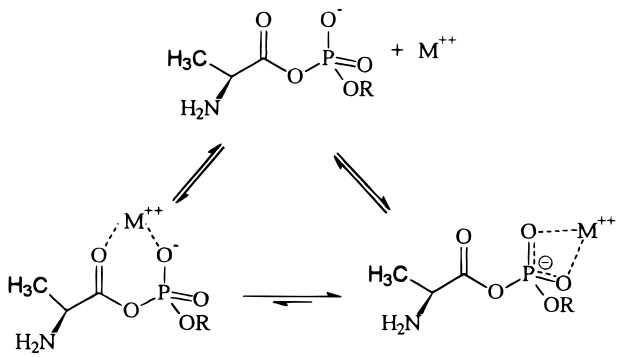
water reaction at pH > pK_a of the amino group is about 2500-fold faster at the lower temperature (or ca. 25 000 times faster at the same temperature) for alanyl ethyl phosphate. This suggests that the N-protonated species reacts with hydroxide. Finally, the hydroxide dependent rate of hydrolysis of benzoyl methyl phosphate at 58 °C exceeds that of alanyl ethyl phosphate at 25 °C by the smallest ratio, consistent with a minimal effect of the neutral amino group on the adjacent anion.

Formation of Amides. The free amino group of alanyl ethyl phosphate or alanine can add to another alanyl ethyl phosphate to form a dipeptidyl molecule. Oligomeric products can result from further reactions of alanyl ethyl phosphate and the dipeptide. We observe no products that retain the acyl phosphate functionality although the amino group is available in alanyl ethyl phosphate. The pK_a of the amino group of alanyl ethyl phosphate is 2 units lower than that of alanine (9.9). Hence, there should be much more alanyl ethyl phosphate than alanine in its nucleophilic amine form, but it should be a weaker nucleophile. An alternative explanation for the absence of any dipeptidyl phosphate would be an undetected intramolecular reaction that forms an oxazolone[14] or diketopiperazine.[15] Oligomerization is a second-order process and minimized in dilute solutions, as reported for phenylalanyl adenylate.[16] We have investigated the general use of N-protected aminoacyl alkyl phosphates as reagents for peptide synthesis in water. They give reasonable yields, and no loss of chiral integrity can be detected at the stereocenters.[3,17] This is presumably the result of the anionic character of the material suppressing enolate formation.[17]

Reactions with Alcohols. Since alanyl ethyl phosphate reacts slowly with water, it is not surprising that it undergoes solvolysis slowly in methanol. The alcohol can react by a similar mechanism with the transfer of the hydroxyl proton in the intermediate. The less polar solvent should discourage the more reactive ionic forms. The reaction of alanyl ethyl phosphate with 2-propanol was not observed to proceed at all toward the ester. This is consistent with its being even less polar and more hindered.

Metal Ion Catalysis. The results in Table 2 show that one of the major differences between metals is their affinities for alanyl ethyl phosphate. The rate constants for the complexes (at saturation) are not related to the affinity, but both contribute to the effective rate. The phosphate anion provides a localized site for binding. This position is not particularly effective for promoting hydrolysis since stabilizing the carbonyl tetrahedral intermediate and its associated transition states is most critical (Scheme 6). If the binding were completely localized at the phosphate anion, catalysis would likely be due to a decreased electrostatic barrier.

Scheme 6



The amino group may serve as a template to direct binding in this direction. Thus, catalysis can be accomplished by a small portion of the complex being bidentate-coordinated (to the phosphate and oxyanion of the intermediate and related transition states).[18] The use of specific ligands on the metal may further facilitate such coordination.

Conclusions

Acid, base, and metal ions enhance the reaction of water with alanyl ethyl phosphate. The kinetic patterns are consistent with reactions involving intermediates that undergo proton transfers. Reactions with alcohols are quite slow and will require the design of catalysts in order to be useful. One potential application for such reactions could be in producing synthetically aminoacylated t-RNAs to make proteins with unnatural amino acid derivatives in their backbone.[19]

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