Metal-Ion Facilitated Hydrolysis of Hippuryl-DL-β-Phenyllactate, a Carboxypeptidase Substrate

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The hydrolysis of hippuryl-DL-β-phenyl-lactate (I), a carboxypeptidase A substrate, has been studied over the pH range 3.7–6.0. The products from the hydrolysis are hippuric acid and DL-β-phenyllactate. The rate of spontaneous hydrolysis of I *obeys the rate law, rate* = $k_o + k_{OH}[OH]$, *with* k_o *of* 2.2×10^{-6} sec^{-1} *and* k_{OH} *of* 4.36×10^3 M^{-1} sec^{-1}. *Of several divalent metal ions examined, Cu(II) provides the greatest increase in hydrolysis rate, with an enhancement of 1200 times the spontaneous rate. The rate acceleration afforded by divalent metal ions is most likely due to a transition-state effect in which the leaving group, β-phenyllactate, is stabilized by chelation to the metal ion. The relevance of these kinetic studies to the mechanism of carboxypeptidase A and other metallohydrolases is discussed.*

Introduction

The function of divalent metal ions in metallohydrolase action continues to be a subject of intense interest and investigation. Useful mechanistic information can be gained from studies of metal ion catalyzed reactions of substrates of hydrolytic enzymes. Metal ion facilitated cleavage of esters [1–5], glycosides and acetals [6, 7] and amides [8–10] has been used to enhance the mechanistic understanding of carboxypeptidase A, alkaline phosphatase, amylase, thermolysin, carbonic anhydrase, urease and other hydrolases. Often the substrates chosen for study are designed so as to contain a coordinating group that can position the metal ion in proximity to the functional group susceptible to hydrolytic attack. In this arrangement the metal ion can be temporarily 'immobilized and oriented' in much the same way as in the enzyme. The chelated metal has the potential to facilitate the hydrolytic reaction in at least three ways: (1) by polarizing the substrate carbonyl of an ester or amide [2]; (2) by stabilizing the leaving group (transition-state effect) [3, 4], or (3) by providing a metal bound hydroxide [2, 8].

The compound, O-(N-benzoylglycyl)-L-β-phenyllactate (hippuryl-L-phenyllactate) (*I*), as well as analogous O-acyl-2-hydroxyacids, are readily hydrolyzed in the presence of carboxypeptidase A [11, 12]. In fact, with a k_{cat} of $28–35 \times 10^3$ min^{-1}, *I* has the highest turnover rate of any known carboxypeptidase A substrate [13]. Even though the enzyme catalyzed hydrolysis of *I* is complicated by two factors, an unusual pH–rate profile [14, 15] and substrate–product inhibition [16, 17], it has gained widespread use in carboxypeptidase A analysis.

We have discovered that the ester bond of *I* is susceptible to metal-ion-facilitated hydrolysis. Screening of Cu(II), Ni(II), Zn(II), Co(II) and Mn(II) indicated that Cu(II) was the most effective catalyst. Reported here is the detailed kinetic analysis of the Cu(II)-facilitated hydrolysis of *I*.

The involvement of metal ions in the hydrolysis of amino acid esters [18] and O-acyl derivatives of naturally occurring, long-chain 2-hydroxyacids [19, 20] has previously been described.

Experimental

Materials

Hippuryl-DL-β-phenyllactic acid (sodium salt), BF_3/MeOH, and BIS-TRIS were obtained from Sigma Chemical Co. The methyl ester of hippurylphenyllactate was prepared as follows [21]: to a solution of hippurylphenyllactate in anhydrous methanol (200 mg, 10 ml) was added 14% BF_3/MeOH (10 ml). The reaction mixture was refluxed for two hours and poured into saturated sodium bicarbonate. Ether extraction yielded white crystals, m.p. 163–4 °C; the i.r. spectrum (KBr), which was very similar to

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that of hippuryllactic acid, ethyl ester*, showed major absorptions at 3250 cm^{-1} (amide, N–H), 1720 cm^{-1} (doublet, ester, C=O) and 1630 cm^{-1} (amide, C=O). The nmr spectrum in $CDCl_3$ was in agreement with the proposed structure: δ7–8 (10 H, multiplet), δ5.3 (1 H, triplet), δ4.2 (2 H, triplet), δ3.7 (3 H, singlet) and δ3.2 (2 H, doublet).

Determination of pK_a

The acid dissociation constant for hippuryl-DL-β-phenyllactic acid was evaluated by potentiometric titration. A plot of pH *vs.* volume of titrant was prepared. An average of three trials resulted in a pK_a of 2.95. This is reasonable as the pK_a of lactic acid is 3.08 [22].

Kinetic Measurements

The rate of production of hippuric acid by hydrolysis of hippurylphenyllactate was monitored by recording the increase in absorbance at 254 nm [16]. Measurements were made on a GCA/McPherson Spectrophotometer, Model EU-707. Solutions of the divalent metal nitrates were made in acetate (pH 3.7–5.7) and BIS-TRIS (pH 5.6–6.0) buffers. A rate measurement at 5.6 in each buffer provided similar results. Catalysis by buffer ions was not noted. Solutions of hippurylphenyllactate were prepared in glass distilled water at a concentration of 0.003 *M*. Solutions of the methyl ester of hippurylphenyllactate were prepared in acetone. The ionic strength of each reaction mixture was maintained at 0.1 *M* by the addition of sodium nitrate. Each run was initiated by the addition of 100 μl of the ester to a thermostatted buffer solution or metal ion solution in a 3 ml cuvette. All concentration levels of ester *I* were saturating. Reactions were monitored to completion as determined by zero absorbance change. Pseudo first order rate constants were calculated from plots of $\ln(A_\infty - A_o/A_\infty - A_t)$ *vs.* time with linear least squares regression analysis. No absorbance change at 254 nm was measured for either spontaneous or metal-promoted hydrolysis of the methyl ester of hippurylphenyllactate at temperatures up to 40°. Hydroxide-ion concentrations at 37 °C were calculated using pK_w = 13.6801.

Product Study

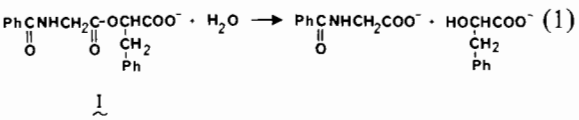
Large scale reactions were conducted to study the products of spontaneous and metal catalyzed hydrolysis of *I*. The concentration ratio of ester to metal was 1:100. The reactants in buffered solutions were incubated at 40 °C for several hours. The light blue or white precipitate which formed during metal facilitated reaction was filtered and confirmed by i.r. analysis to be Cu(β-phenyllactate)$_2$ and Zn-(β-phenyllactate)$_2$ respectively [19]. The filtrate from above or the reaction solution from the non-metal reaction was passed through a Dowex 50-W column (6 × 1 cm) and eluted with 0.1 *N* HCl. Fractions of 1 ml were collected and monitored at 260 nm. The first component (hippuric acid) was eluted with 15 ml of dilute HCl. The reaction mixture from the non-metal reaction also yielded a second component (β-phenyllactate) after elution with 45 ml of dilute HCl. No other products could be detected.

Footnote: *The i.r. spectrum of hippuryllactic acid, ethyl ester, is recorded in Sadtler Standard Spectra, No. 6837, 1962.

Results and Discussion

Product Study

Under the conditions described in the Experimental Section, *I* was hydrolyzed to hippuric acid (N-benzoylglycine) and β-phenyllactate (Reaction 1, Ph = phenyl). The products were separated on an ion-



(1)

I

exchange column and characterized by u.v. spectrophotometry. Hydrolysis of the amide bond of hippuric acid was not observed. The same products were formed during divalent metal promoted hydrolysis of *I* except that β-phenyllactate was complexed to the metal [19]. The products of carboxypeptidase catalyzed hydrolysis of *I* are also, presumably, hippuric acid and β-phenyllactate, but direct experimental evidence is lacking. However, the use of *I* in the spectrophotometric assay of carboxypeptidase is based on the increase in absorbance at 254 nm that occurs when hippuric acid derivatives are subjected to hydrolysis. This absorbance increase has been attributed to the formation of hippuric acid [16, 23]. Both metal promoted and carboxypeptidase-catalyzed hydrolysis of *I* results in cleavage of the ester bond. Since the overall outcome of the two processes is the same, kinetic and mechanistic comparisons should be possible and should add insight to the role of the metal ion in carboxypeptidase A.

Spontaneous Hydrolysis

Values of k_{obs} obtained from the spontaneous hydrolysis of *I* in the pH range 3.7–6.0 are listed in Table Ia. Rate change as a function of pH for the hydrolysis of *I* demonstrates only a small dependence on pH. The apparent rate expression for spontaneous hydrolysis is

$$\text{Rate} = k_o + k_{OH}[OH^-]$$

TABLE I. Spontaneous Hydrolysis and Cu(II)-promoted Hydrolysis of Hippurylphenyllactate as a Function of pH at 37 °C and μ = 0.1 *M*. Ester concentration was 1×10^{-4} *M* and Cu(II) concentration was 0.03 *M*.

(a) Spontaneous hydrolysis

pH	$10^5 k_{obs}$, sec^{-1}
3.7	1.1
4.0	1.3
4.5	1.8
5.0	2.0
5.5	2.4
6.0	3.1

(b) Cu(II)-Promoted hydrolysis

pH	$10^3 k_{obs}$, sec^{-1}
3.7	5.7
4.0	6.5
4.4	7.8
4.7	9.9
5.0	11.9
5.5	15.6
6.0	18.2

TABLE II. Relative Efficiency of Various Metals in the Hydrolysis of Hippurylphenyllactate at 37 °C, pH 4.4 and μ = 0.1 *M*. Ester concentration was 1×10^{-4} *M*, and metal concentration was 0.02 *M*.

Metal	$10^3 k_{obs}$, sec^{-1}	Relative Rate
Cu(II)	7.8	1.0
Zn(II)	1.2	0.15
Ni(II)	0.8	0.10
Mn(II)	0.5	0.06
Co(II)	0.5	0.06

where k_o and k_{OH} are the rate constants for water and hydroxide ion attack, respectively. Analysis of a plot of log k_{obs} *vs.* pH yielded $k_o = 8.6 \times 10^{-4}$ sec^{-1} and $k_{OH} = 4.36 \times 10^3$ M^{-1} sec^{-1}. At pH values greater than 9 very rapid hydrolysis of *I* was noted in our experiments and those of others [24]. Because compound *I* is relatively labile at pH values less than its pK_a (2.95), it is not possible to directly evaluate the influence of the unionized carboxyl group on the spontaneous hydrolysis. However, we did note that hydrolysis of the methyl-ester of *I* was not detectable in the pH range of 3.7–6.0. This would provide confirmation that the carboxylate anion must have some involvement in the spontaneous hydrolysis. The participation of intramolecular carboxyl groups in ester hydrolysis has been extensively investigated from a kinetic standpoint [4, 25].

TABLE III. Kinetic Constants of the Cu(II)-promoted Hydrolysis of *I*. The constants are the results of measurements at pH 4.4, 37 °C, μ = 0.1 *M*, [Cu(II)] under saturation conditions and ester concentration of 1.0×10^{-4} *M*. See text for definitions of constants.

Kinetic Constant	Value
k_o	8.6×10^{-4} sec^{-1}
k_{OH}	5.6×10^6 M^{-1} sec^{-1}
k_{obs}/α	18 sec^{-1}
k_{OH} (Cu(II))/k_{OH} (spont.)	1.28×10^3
K_{form}	29 m*M*

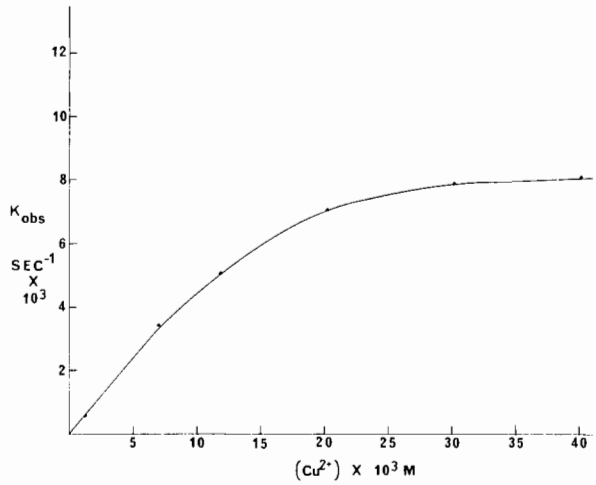


Fig. 1. Plot of k_{obs} *vs.* Cu(II) concentration for hydrolysis of hippurylphenyllactate at pH 4.4, 37 °C and μ = 0.1 *M*. Ester concentration was 1.0×10^{-4} *M*.

Metal Promoted Hydrolysis

Hippuryl phenyllactate, in the presence of divalent metal ions, is rapidly hydrolyzed to hippuric acid and metal(II) (β-phenyllactate)$_2$. The Cu(II), Zn(II), Ni(II), Mn(II) and Co(II)-promoted reactions were investigated in the pH range 3.7–6.0 at 37 °C and an ionic strength of 0.1 *M* (sodium nitrate) (Tables Ib and II). Increasing metal ion concentration with constant but saturating substrate concentration led to a constant reaction rate only with Cu(II) (Fig. 1). A reaction rate independent of Cu(II) concentration was not attained at Cu(II): *I* ratios less than 400:1. With Cu(II) at pH 4.4, T = 37 °C, k_{obs} makes an asymptotical approach to 9×10^{-3} sec^{-1}. A plot of $1/k_{obs}$ *vs.* 1/[Cu(II)] yields a straight line from which the formation constant, K_{form}, for the com-

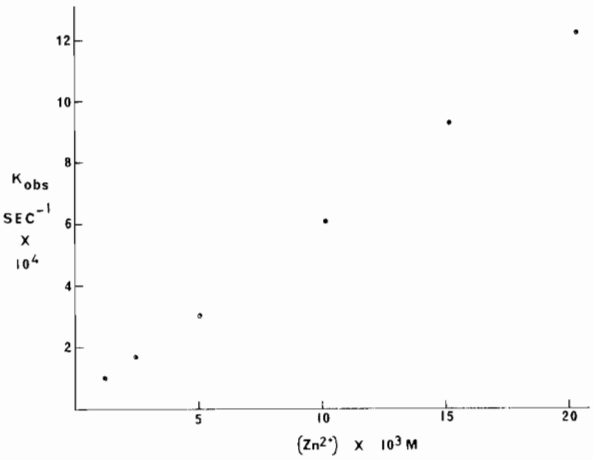


Fig. 2. Plot of k_{obs} *vs.* Zn(II) concentration for hydrolysis of hippurylphenyllactate at pH 4.4, 37 °C and μ = 0.1 *M*. Ester concentration was 1.0×10^{-4} *M*.

plex of Cu(II) with *I* can be determined. Table III records several kinetic characteristics of the Cu(II)-promoted hydrolysis of *I*. A kinetic determination of k_o and k_{OH} was possible from a plot of log k_{obs} *vs.* pH. These values also are listed in Table III.

The rate constant for intramolecular hydroxide-ion attack (metal-bound hydroxide), k, may be calculated from the relationship

$$k = \frac{k_{obs}}{\alpha}$$

where α is the degree of ionization at the pH of measurement [3]. At pH 4.4, under saturation conditions, and assuming the pK_a for aquacopper(II) ligands as 7.5 [26], k is calculated to be 18 sec^{-1}. This is comparable to a k value of 50 sec^{-1} for Cu(II)-promoted hydrolysis of 8-acetoxy-quinoline-2-carboxylic acid [3].

Plots of k_{obs} *vs.* [Zn(II)] (Fig. 2) or [Ni(II)] were obtained at metal concentrations up to 0.02 *M* (200-fold excess over substrate). Higher concentrations of Zn(II) and Ni(II) were not possible because of precipitation. The necessity for high concentration ratios of metal ion to substrate is indicative of weak coordination of ester *I* to the metal ions. Except for Cu(II), there is probably only a small percentage of complexation between metal ion and substrate in the buffered solutions. Two structures, A and B, can be drawn to represent the coordination complex formed in these experiments (Fig. 3). Coordination of the carboxylate anion is similar in both structures but coordination of the carbonyl oxygen of the ester functional group (B) or coordination of the ether-type oxygen of the ester group (A) leads to a 7- or 5-membered cyclic intermediate, respectively.

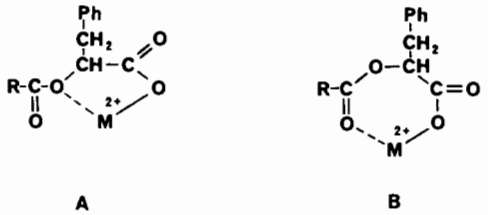


Fig. 3. Structures A and B represent two possible modes of coordination of hippurylphenyllactate to a divalent metal ion. R = $PhCONHCH_2$-, M = metal.

In spite of the weak interaction between transition metals and hippuryl phenyllactate, the rate enhancements are observed for the hydrolysis of the ester group in the presence of divalent metal ions. The most effective conditions studied, (Cu(II) at T = 37 °C, pH 4.4) caused a rate acceleration of base hydrolysis of *I* about 1200 times greater than the spontaneous rate (Table III). At a metal ion concentration of 0.02 *M*, the other metals were much less effective as catalysts (Table II). The order of reactivity, Cu(II) $\gg$ Zn(II) > Ni(II) > Mn(II) = Co(II) is typical for metal catalyzed hydrolysis of esters [3]. At least one exception to this order has been reported. Zn(II) is more effective than Cu(II) in promoting the hydrolysis of 8-acetoxyquinoline-2-carboxylic acid [4].

In previous studies, we have reported Cu(II) [19] and Zn(II) [20] facilitated hydrolysis of O-acyl-2-hydroxyacids. From these investigations we concluded that intermediates similar to A in Fig. 3 are more likely than B. One especially attractive feature of structure A is that it allows the metal ion to perform a dual role: (1) increase the electropositive nature of the carbonyl carbon atom by electron withdrawal and (2) stabilize the leaving group (β-phenyllactate) by chelation. The divalent metal ion. The final product, β-phenyllactate, is able ester bond by strong coordination to the carboxylate ion and weak coordination to the ether-type oxygen. However, as the C–O bond begins to break, the developing negative charge on the oxygen atom may be stabilized by strong coordination to the metal ion. The final product, β-phenyllactate, is able to bind strongly to divalent metal ions, forming a stable complex [19].

Mechanism of Carboxypeptidase A

Structures A and B in Fig. 3 may represent the mode of binding of hippuryl-β-phenyllactate and other ester substrates to the zinc ion at the active site of carboxypeptidase A. The manner in which peptide substrates bind to carboxypeptidase A has been elucidated, but all known ester substrates are hydrolyzed too rapidly to permit X-ray diffraction

studies of enzyme substrate interaction. The binding of peptide substrates most likely proceeds by displacement of water from Zn(II) leading to coordination of the amide carbonyl [27]. Auld and Holmquist have suggested that the active-site zinc atom is more important in the binding of ester substrates than peptide substrates [28]. Their reasoning is threefold: (1) interchange of metals at the active site of the enzyme greatly affects the binding of esters but not the rate of hydrolysis. The reverse is true for peptide substrates. (2) The apoenzyme of carboxypeptidase A binds esters much more weakly than peptide substrates. (3) Phenylacetate and 2-phenylpropionate inhibit the binding of esters to carboxypeptidase A, presumably by competing for the active site metal. Contrary to these compelling arguments, more recent reports on the mechanism of action of carboxypeptidase A in ester hydrolysis propose that zinc coordination to the carbonyl oxygen of the ester substrate does not lead to productive binding [27, 29, 30]. The suggested primary function of the active-site zinc ion then is to offer a metal bound hydroxide group for nucleophilic attack on the ester carbonyl.

The reaction system we have described, divalent metal-facilitated hydrolysis of hippurylphenyllactate, a carboxypeptidase A substrate, may prove to be a useful model for a continuing investigation of the mechanism of carboxypeptidase and other metallohydrolases. The model reaction provides not only a representation of possible substrate–active-site metal interactions, but also offers the opportunity to measure the importance of metal ion stabilization of the leaving group, *i.e.* transition state effect in metallohydrolase-catalyzed reactions.

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