Substituent Effects in the Carboxypeptidase A Catalyzed Hydrolysis of Substituted L,β-Phenyllactate Esters†

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ABSTRACT: The carboxypeptidase A catalyzed hydrolysis of an extensive series of substituted cinnamoyl-L,β-phenyllactate esters has been investigated. Plots of k_{cat} vs pH are sigmoidal in the pH range 5−9 with an average apparent pK_a^{ES} of 6.6 ± 0.1. The values of K_m are pH independent in the range pH 5−8. Plots of log k_{cat}/K_m vs pH give pK_a^E values of 6.4 and 9.0 that do not vary significantly through the series. A plot of log k_{cat} (pH 8) vs σ, the Hammett substituent constant, is linear with a slope ϱ of 0.5, while log K_m vs σ has a slope of −0.4. The plot of log k_{cat}/K_m vs σ is also linear with $\varrho = 0.9$. The Hammett plots are linear at both pH 6 and 8 with closely similar slopes, which indicates that the apparent pK_a^{ES} near pH 6 does not reflect a change in the rate-determining step. The enzymatic reactions and the nonenzymatic OH^- catalyzed hydrolysis reactions are affected alike by changes in the substituent groups; a plot of log k_{OH}, the second-order rate constant for alkaline hydrolysis of the esters, vs log k_{cat}/K_m is linear with a slope of 0.9. There is little effect of changing the substituent group in the nonenzymatic pH-independent hydrolysis of the Zn(II) complex of corresponding 4-substituted cinnamic acid 6-carboxypicolinic acid anhydrides ($\varrho \leq 0.1$). The carboxypeptidase A catalyzed hydrolysis of *O*-(α-benzoylamino)cinnamoyl-L,β-phenyllactate at pH values from 7.5 to 9.5 produces no absorbance changes in the range 340−370 nm that would be characteristic of the oxazolinone formed by attack of the neighboring benzoylamino group on an anhydride intermediate. Also, there is only a small effect of high concentrations of fluoride ion on the reactions of that ester. The above results do not support a mechanism involving rate-determining hydrolysis of an anhydride intermediate in the reactions of the benzoylamino derivative. If an anhydride occurs as an intermediate in the reactions of the 4-substituted cinnamoyl esters, then it does not build up. A mechanism involving the attack of Zn(II)-bound OH^- on the ester substrates is considered as an alternative to nucleophilic attack by Glu-270.

Carboxypeptidase A (peptidyl L-amino acid hydrolase, EC 3.4.12.2) (CPA)[1] is a Zn(II) metalloenzyme that catalyzes the hydrolysis of carboxyl terminal peptides and O esters of L,β-phenyllactic acid and mandelic acid (Kaiser & Kaiser, 1972; Hartsuck & Lipscomb, 1971). The X-ray crystallographic analysis of the enzyme at 2-Å resolution revealed the presence of the Zn(II) ion and the γ-carboxyl group of Glu-270 in the active site (Lipscomb, 1970; Ludwig & Lipscomb, 1973). Mechanisms were suggested for peptide hydrolysis involving nucleophilic or general base catalysis by Glu-270, and it was assumed that similar mechanisms would occur in reactions of ester substrates. It was later established that the initial binding sites for peptides and esters are differ it (Auld & Holmquist, 1974; King et al., 1987), but that does not preclude similar mechanisms. Kinetic evidence has been presented for the occurrence of an intermediate in the CPA catalyzed hydrolysis of substituted cinnamoyl-L,β-phenyllactate esters (Makinen et al., 1976, 1979; Suh et al., 1985). This intermediate was assumed to be an anhydride of Glu-270, but that has not been unambiguously established [see also, Sander and Witzel (1985) and Britt and Peticolas (1992)].

The k_{cat} vs pH profiles for CPA catalyzed hydrolysis of β-phenyllactate esters are sigmoidal with $pK_{app} \sim 6$ (Hall et al., 1969; King & Fife, 1983). Values of pK_1^E and pK_a^{ES} are quite similar. Comparable pK_{app} values have also been obtained in peptide hydrolysis (Auld & Vallee, 1970, 1971). The pK_{app} near 6 in plots of log (k_{cat}/K_m) vs pH has been assigned to Glu-270 (Hartsuck & Lipscomb, 1971; Suh & Kaiser, 1976; Auld & Vallee, 1970; Kaiser & Kaiser 1972), and this is supported by chemical modification of the enzyme (Petra, 1971; Hass & Neurath, 1971). This is consistent with Glu-270 involvement in the reaction with the pK_{app} being the pK_a of the γ-carboxyl group. However, the pK_a^{ES} for mandelate esters is >7 (Carson & Kaiser, 1966; King & Fife, 1983), and k_{cat} for the hydrolysis of *O*-hippuryl-L,β-phenyllactate is pH independent in the pH range 5−10 (Bunting et al., 1974). Furthermore, the ΔH_i^{ES} of ~7 kcal/mol is not in accord with the ionization of a carboxyl group (Makinen et al., 1979; Auld & Vallee, 1971).[2]

The apparent pK_a^{ES} near pH 6 does not necessarily reflect the pK_a of a functional group; it could also result from a change in the rate-limiting step, or it could be a composite constant (Bruice & Schmir, 1959; King & Fife, 1983). Understanding the nature of the apparent pK_a is central to the problem of the mechanism of the reaction. The

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[1] Abbreviations: CPA, bovine pancreatic carboxypeptidase A; CPL, *O*-(*trans*-cinnamoyl)-L,β-phenyllactic acid; Tris, tris(hydroxymethyl)-aminomethane.

[2] A perturbed ΔH_i could, of course, result because of the environment of the active site as compared with aqueous solution. Makinen et al. (1979) suggested that the pK_a of the Zn(II)-bound water molecule is close to 6, but see King and Fife (1983).

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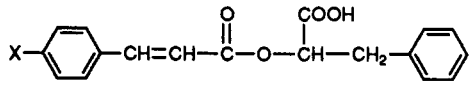
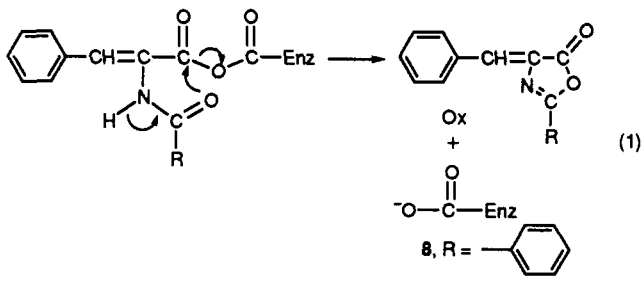
determination of substituent effects in the ester substrate should allow this understanding. For example, if pK_{app}^{ES} represents a change in the rate-determining step, then a plot of log k_{cat} at pH 6 vs σ, the Hammett substituent constant (Hammett, 1940), would have a different slope than similar plots at high pH and could show downward curvature for reactions in which a substituent in the acyl group of cinnamoyl-L,β-phenyllactate esters is varied. On the other hand, if pK_{app}^{ES} is a composite constant, then its value might vary significantly with changing substituents. Kaiser et al. (1974) have previously studied several 4-substituted cinnamoyl-L,β-phenyllactate esters, but at only one pH value (7.5). We have therefore determined k_{cat} and k_{cat}/K_m vs pH profiles for CPA-catalyzed hydrolysis of the esters **1**−**7** in the range pH 5−10. Hammett $\sigma\rho$ plots have been made at both pH 6.0 and 8.0, spanning pK_{app}. The Hammett plot for k_{cat} has been compared with that in the nonenzymatic hydrolysis of the Zn(II) complexes of substituted cinnamic acid 6-carboxypicolinic acid anhydrides.



1, X = $N(CH_3)_2$
2, X = OiPr
3, X = OCH_3
4, X = CH_3
5, X = H
6, X = Cl
7, X = NO_2

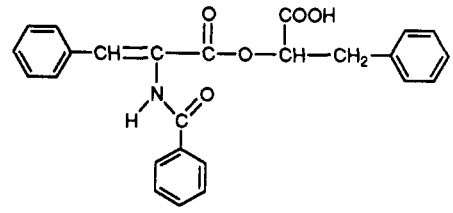
If an anhydride intermediate is produced from an α-(*N*-acylamino)cinnamoyl ester, then an oxazolinone (Ox) would be formed by a subsequent nucleophilic attack of the neighboring *N*-acylamino group. Such oxazolinones have



(1)

8, R =

strong characteristic absorbance at 340−370 nm and are easily identified (Suh et al., 1985; Fife et al., 1988; de Jersey et al., 1966). Neighboring acetamido and benzamido groups are highly effective intramolecular nucleophiles toward esters with good leaving groups (Fife et al., 1988; de Jersey et al., 1966). Suh et al. (1985, 1986) investigated the CPA-catalyzed reactions of α-(*N*-acylamino)cinnamate esters and did not detect any oxazolinone at pH 7.5. Nevertheless, a nucleophilic mechanism was suggested. Cyclization reactions of that type are OH^- catalyzed (Fife et al., 1988) and would be more competitive with the enzymatic hydrolysis at higher pH. We have, therefore, also carried out a thorough search for an oxazolinone (**8**) in the CPA-catalyzed reactions of **9** under conditions that would maximize the likelihood of its detection. Suh et al. (1985) considered that an anhydride intermediate builds up when **9** is the substrate. The results presented in this paper make such postulations quite unlikely.



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EXPERIMENTAL SECTION

Materials. All substituted cinnamoyl-L,β-phenyllactate esters were prepared by the method of Tomalin et al. (1970). The sodium salts of the esters were generally prepared as follows for *O*-(*p*-nitrocinnamoyl)-L,β-phenyllactate. The ester was titrated with 0.1 M NaOH. The pH was maintained below pH 7 throughout the titration. The mixture was then filtered, and the water was removed by rotary evaporation. Absolute ethanol was added to the oily residue. The ethanol and residual water were removed by rotary evaporation. Warm acetone was added to the remaining oil, and the solution was filtered. The filtrate was rotary evaporated. The residue was stirred overnight in ether. The solid material was then vacuum dried. After titration and lyophilization, the sodium salts of esters **2**−**4** and **6** were recrystallized from water containing NaCl. The final products were white solids.

Hippuryl-L-phenylalanine was purchased from Sigma. 2-Phenyl-4-benzylideneoxazolin-5-one (**8**) was prepared according to Gillespie and Snyder (1943). *O*-(α-Benzoylamino)cinnamoyl-L,β-phenyllactic acid (**9**) was prepared by the method of Suh et al. (1985). 4-Substituted cinnamic acid 6-carboxypicolinic acid anhydrides were prepared by the method of Fife and Przystas (1983).

Carboxypeptidase A (Sigma Lot No. C-9762 or 20H-8000) was dialyzed in 0.05 M Tris-HCl buffer (μ = 0.5 M with NaCl, $[Zn^{2+}] = 10^{-4}$ M) pH 7.5, at 5 °C for 36 h. The buffer solution was changed after 18 h. After dialysis, the clear enzyme solution was centrifuged at 15 000 rpm for 15 min at 5 °C. The supernatant was filtered through Millipore Millex filters and stored at 5 °C. The activity of the enzyme stock solution was routinely checked with ester substrate **5** and/or hippuryl-L-phenylalanine at pH 7.5. The protein concentration was determined from the extinction coefficient at 278 nm, $\epsilon = 6.42 \times 10^4$ M^{-1} cm^{-1} (Simpson et al., 1963).

Kinetic Measurements. Nonenzymatic reaction rates of **1**−**7** were measured with a Beckman Model 25 spectrophotometer at 30 °C. The reactions were monitored at a predetermined wavelength by following the disappearance of the reactant. The chosen wavelengths were those which afforded a maximum difference between initial and final absorbance. The extinction coefficients were determined with a Pye-Unicam Model SP8-100 spectrophotometer at 30 °C. Each run was initiated by adding 20 μL of ester stock solution in water to 2 mL of buffer solution in the cuvette. The ionic strength was invariably 0.5 M with NaCl. Pseudo-first-order rate constants (k_{obsd}) were calculated using a nonlinear regression computer program. The pH of the reaction solutions was measured with a Radiometer PHM 22r pH meter at 30 °C. The ion product of water at 30 °C was taken to be 1.47×10^{-14}.

The rates of hydrolysis of the mixed anhydrides of 6-carboxypicolinic acid and substituted cinnamic acids were measured with a Pye-Unicam SP8-100 or a Durrum D-110

Table 1: Second-Order Rate Constants (k_{OH}) for Hydroxide Ion-Catalyzed Hydrolysis of Substituted Cinnamoyl-L,β-phenyllactate Esters at 30 °C (μ = 0.5 M with NaCl)

ester	σ	k_{OH} (M^{-1} s^{-1})
1	−0.83	3.95×10^{-3}
2	−0.45	6.26×10^{-3}
3	−0.268	6.80×10^{-3}
4	−0.17	9.80×10^{-3}
5	0	1.51×10^{-2}
6	0.227	1.93×10^{-2}
7	0.778	1.22×10^{-1}

stopped-flow spectrophotometer employing methods described in Fife and Przystas (1983). Buffer catalysis was observed in reactions in the absence of metal ions. Therefore, the values of k_{obsd}, the pseudo-first-order rate constant, were determined by extrapolation to zero buffer concentration in the buffers chloroacetate, formate, and lutidine. Hydrochloric acid solutions were employed at pH < 3. The solvent employed for hydrolysis of the mixed anhydrides was 50% dioxane−water (v/v) because of limited water solubility of some of the compounds.

In carrying out enzymatic rate measurements, the enzyme stock solution was diluted by adding 100 μL to 4.9 mL of 0.05 M Tris-HCl buffer (μ = 0.5 M NaCl, pH 7.5). Enzymatic rates were measured with a Pye-Unicam SP8-100 spectrophotometer at 30 °C, except those of **9**, which were determined with a Beckman DU 7500 spectrophotometer. The pH range studied was 5.0−10.3 with all esters. All buffer components were reagent-grade materials, and deionized water was used throughout. The buffers employed were Tris−HOAC at pH 5.5−6.5, Tris-HCl at pH 7.0−8.5, ammediol hydrochloride at pH 9.0−10.0, and $NaHCO_3$ at pH 10.3. The procedure was that followed by King and Fife (1983) and Hall et al. (1969). The activity check of the diluted enzyme solution was carried out before and after the enzymatic rate measurements with **4** as the substrate by following the absorbance change at 322 nm at pH 7.5. Initial rates were obtained in the kinetic runs, and Eadie−Hofstee plots of V vs $V/[S_0]$ were used to determine k_{cat} and K_m. At all pH values, the substrate concentrations bracketed the apparent K_m. Initial rate data were always corrected for spontaneous hydrolysis.

Stock solutions of **8** were made in acetonitrile. However, **8** was only soluble in the buffers at a concentration of 1.8×10^{-5} M if the acetonitrile concentration was at least 30%. Stock solutions of **9** were also made in acetonitrile. The concentration of acetonitrile in the reaction cuvette was then 1%. Initial velocity measurements were the same in the presence or absence of this amount of acetonitrile.

RESULTS

The alkaline hydrolysis of the substituted cinnamoyl-L,β-phenyllactate esters (**1**−**7**) gives a linear relationship between log k_{obsd} and pH with a slope of 1.0. The second-order rate constants k_{OH} are summarized in Table 1. In Figure 1, the plot of log k_{OH} vs σ, the Hammett substituent constant (Hammett, 1940), is presented. The slope (ϱ) is 0.93 with a correlation coefficient of r = 0.97.

The parameters k_{cat} and K_m for the CPA-catalyzed hydrolysis of **1**−**7** were determined from the linear plots of V vs $V/[S_0]$ at each pH. The log k_{cat} vs pH profiles for all of the esters were hyperbolic in shape. A typical profile (for the 4-nitrocinnamoyl ester **7**) is shown in Figure 2; k_{cat} is pH independent at pH <9.5. With the other esters, k_{cat} begins to increase between pH 9 and pH 9.5. The plots were computer fitted using eq 2 to determine the apparent pK_a^{ES}

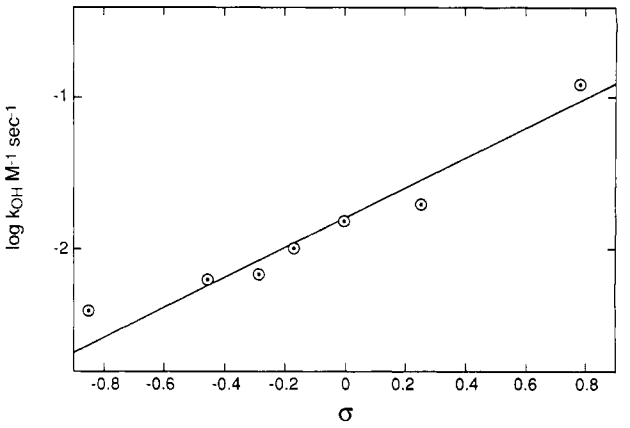


FIGURE 1: Plot of log k_{OH} vs σ for hydroxide ion-catalyzed hydrolysis of substituted cinnamoyl-L,β-phenyllactate esters at 30 °C and μ = 0.5 M with NaCl.

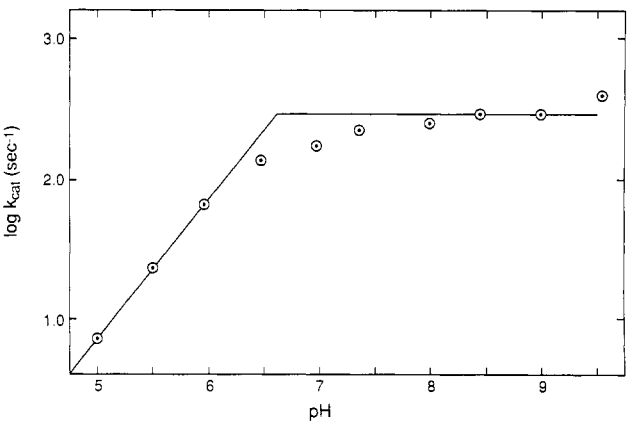


FIGURE 2: Plot of k_{cat} vs pH for CPA-catalyzed hydrolysis of *p*-nitrocinnamoyl-L,β-phenyllactate at 30 °C and μ = 0.5 M with NaCl.

Table 2: Apparent pK_a^{ES} Values and pH-Independent k_{cat} Values for CPA-Catalyzed Hydrolysis of Substituted Cinnamoyl-L,β-phenyllactate Esters at 30 °C (μ = 0.5 M with NaCl)

ester	pK_a^{ES}	k_{cat}(lim) (s^{-1})
1		40.7
2	6.7	184
3	6.6	83.3
4	6.4	87.9
5	6.3	181
6	6.8	240
7	6.6	275

$$k_{cat} = k_{cat}(\text{lim})\left[\frac{K_a^{ES}}{K_a^{ES} + a_H}\right] \quad (2)$$

and the limiting k_{cat} for the pH-independent portion of the profiles. These constants are given in Table 2. The reported pK_a^{ES} has a calculated accuracy of ± 0.1 pK_a unit, while the k_{cat} values are accurate to ± 3−10%. The K_m values for **2**−**7** are pH-independent from pH 5 to 8 and increase with increasing pH at pH >9. A typical plot of K_m vs pH for the reaction of cinnamoyl-L,β-phenyllactate was shown in King and Fife (1983).

Table 3: Values of k_{cat}, K_m, and k_{cat}/K_m for CPA-Catalyzed Hydrolysis of Substituted Cinnamoyl-L,β-phenyllactate Esters at 30 °C (pH 8.0, μ = 0.5 M with NaCl)

ester	k_{cat} (s^{-1})	$K_m \times 10^4$ (M)	$k_{cat}/K_m \times 10^{-5}$ (M^{-1} s^{-1})
1	37.6	5.09	0.73
2	172	6.90	2.49
3	92.1	2.55	3.61
4	83.8	2.60	3.22
5	169	1.69	10.0
6	239	1.79	13.4
7	253	1.26	20.1

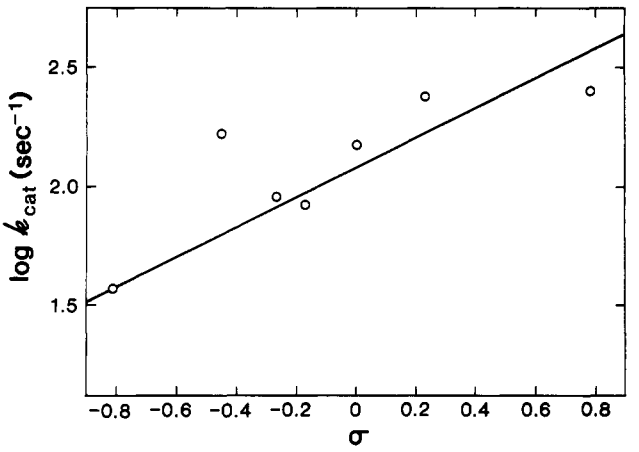


FIGURE 3: Plot of log k_{cat} (pH 8.0) vs σ for CPA-catalyzed hydrolysis of substituted cinnamoyl-L,β-phenyllactate esters at 30 °C and μ = 0.5 M with NaCl.

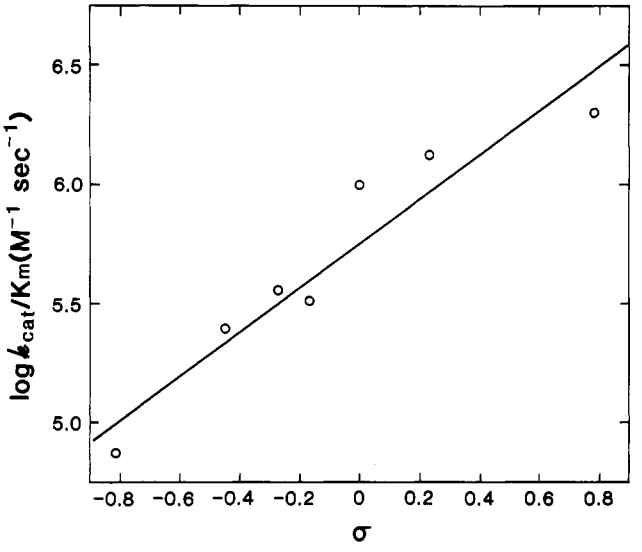


FIGURE 4: Plot of log k_{cat}/K_m (pH 8.0) vs σ for CPA-catalyzed hydrolysis of substituted cinnamoyl-L,β-phenyllactate esters at 30 °C and μ = 0.5 M with NaCl.

Since the apparent pK_a^{ES} values are in the range of 6.3–6.8, a pH of 8.0 was chosen for further analysis of the pH-independent reaction governed by k_{cat}(lim). The k_{cat} values for the esters at pH 8.0 agree reasonably with the calculated values of k_{cat}(lim). The values of k_{cat}, K_m, and k_{cat}/K_m at pH 8.0 are given in Table 3. The Hammett plot of log k_{cat} at pH 8.0 vs σ is shown in Figure 3, and the plot of log k_{cat}/K_m vs σ is shown in Figure 4. The slopes (ϱ) are 0.5 (r = 0.81) and 0.92 (r = 0.95), respectively. The point for *p*-isopropoxy shows considerable positive deviation in the k_{cat} plot (Figure 3) and corresponding positive deviation in the plot of log K_m vs σ (not shown) but a good fit to the line of Figure 4.

Table 4: Values of k_{cat}, K_m, and k_{cat}/K_m for CPA-Catalyzed Hydrolysis of Substituted Cinnamoyl-L,β-phenyllactate Esters at 30 °C (pH 6.0, μ = 0.5 M with NaCl)

ester	k_{cat} (s^{-1})	$K_m \times 10^4$ (M)	$k_{cat}/K_m \times 10^{-5}$ (M^{-1} s^{-1})
2	29.7	4.16	0.71
3	21.6	1.38	1.56
4	31.2	1.93	1.62
5	55.9	1.87	2.99
6	42.2	0.98	4.32
7	66.4	0.97	6.82

Table 5: Slopes of Linear Plots in CPA-Catalyzed Hydrolysis of Substituted Cinnamoyl-L,β-phenyllactate Esters at 30 °C (μ = 0.5 M with NaCl)

plot	slope	r
log k_{cat} vs σ at pH 8.0	+0.5	0.81
log K_m vs σ at pH 8.0	−0.4	0.86
log k_{cat}/K_m vs σ at pH 8.0	+0.92	0.95
log k_{cat} vs σ at pH 6.0	+0.4	0.82
log k_{cat}/K_m vs σ at pH 6.0	+0.76	0.94
log k_{cat}/K_m vs log k_{OH}	+0.87	0.87

Table 6: Rate Constants for pH-Independent Hydrolysis of 6-Carboxypicolinic Substituted Cinnamic Acid Anhydrides at 30 °C and μ = 0.1 M (with KCl) in 50% Dioxane–H_2O (v/v)

substituent	k_o (s^{-1}), no metal ion	k_o (s^{-1}), saturating Zn^{2+}
4-OCH_3	1.1×10^{-3}	2.5×10^{-1}
4-CH_3	1.4×10^{-3}	2.3×10^{-1}
H[a]	1.6×10^{-3}	2.3×10^{-1}
4-Cl	1.8×10^{-3}	2.3×10^{-1}
3-NO_2	2.8×10^{-3}	3.3×10^{-1}

[a] Fife and Przystas (1983).

This may indicate a hydrophobic binding interaction of that substituent, which influences both k_{cat} and K_m. A plot of log K_m vs the Hansch hydrophobicity constants π (Fugita et al., 1964) was reasonably linear with a slope of 0.4 and a correlation coefficient of 0.6. However, correlation of k_{cat} with π was not evident.

The values of k_{cat}, K_m, and k_{cat}/K_m at pH 6.0 are given in Table 4. There is again an excellent linear relationship of these constants with σ. The plot of log k_{cat}/K_m vs σ has a slope of 0.76 with an r of 0.94. A summary of the slopes of the various plots is provided in Table 5. Inclusion of π in 4-parameter equations did not appreciably improve the correlations.

Pseudo-first-order rate constants (k_{obsd}) were determined for hydrolysis of a series of 6-carboxypicolinic acid-substituted cinnamic acid anhydrides in 50% dioxane–water (v/v) at 30 °C (μ = 0.1 M with KCl) in the pH range 1–6. The reactions are pH independent in that pH range. Rate measurements were not carried out at higher pH values because of precipitation of the metal ion. Zinc(II) binds strongly to the anhydrides, and plots of k_{obsd} vs [Zn^{2+}] at constant pH are hyperbolic. Limiting rate constants at a saturating 0.01 M concentration of Zn^{2+} (k_o) are given in Table 6. In all reactions, the ratio of metal ion to anhydride was greater than 100-fold to ensure the formation of a 1:1 complex. Figure 5 shows a plot of log k_o vs σ. There is little effect of electron withdrawal on ϱ in the presence or absence of the metal ion ($\varrho \leq 0.1$).

The CPA (1.8×10^{-8} M) catalyzed hydrolysis of **9** (25 °C) gave k_{cat} values of 0.51 s^{-1} at pH 7.5 and 9.0. Absorbance characteristic of the oxazolinone **8** (λ_{max} =

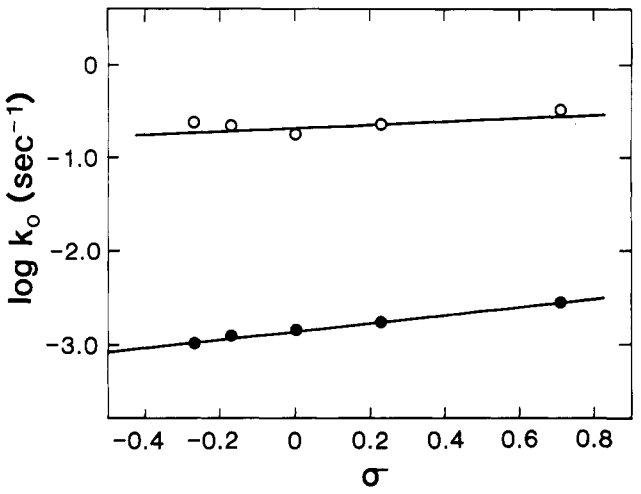


FIGURE 5: Plot of log k_o vs σ for pH-independent hydrolysis of 6-carboxypicolinic acid-substituted cinnamic acid anhydrides in 50% dioxane−H_2O at 30 °C and μ = 0.1 M with KCl in the presence of a saturating 0.01 M concentration of Zn^{2+} (○) and in the absence of metal ion (●).

45 000 M^{-1} cm^{-1} at 359 nm) was not found at pH values from 7.5 to 9.5. The water solubility of **8** is low. The possible presence of any water-insoluble cyclization product was eliminated by adding acetonitrile (up to 50%) to the reaction mixture upon completion of the reaction; no evidence for the presence of **8** was found under these conditions. CPA (2.3×10^{-6} M) was also incubated with **9** at 0 °C at different pH values which ranged from 7.8 to 9.2. After 20 s, precooled acetonitrile was added to give a 50% solution. The spectra failed to show the presence of any **8** but were identical with those of the hydrolysis products of **9**. **8** is reasonably stable under the above conditions, and its characteristic absorbance can be detected when an authentic sample is added separately. The CPA-catalyzed reaction of **9** was also carried out in the presence of 30% acetonitrile, in which **8** is soluble to the extent of 1.87×10^{-5} M. Initial velocities were the same as in water, and again **8** was not detected.

The oxazolinone **8** does not react with the enzyme. **8** and CPA were incubated in Tris-HCl buffer at pH 7.5, μ = 0.5 M with NaCl, at 23 °C for 10 min. Acetonitrile was then added, but there was no significant modification of the spectrum of **8**. The same result was obtained with a reaction medium containing 30 and 50% acetonitrile.

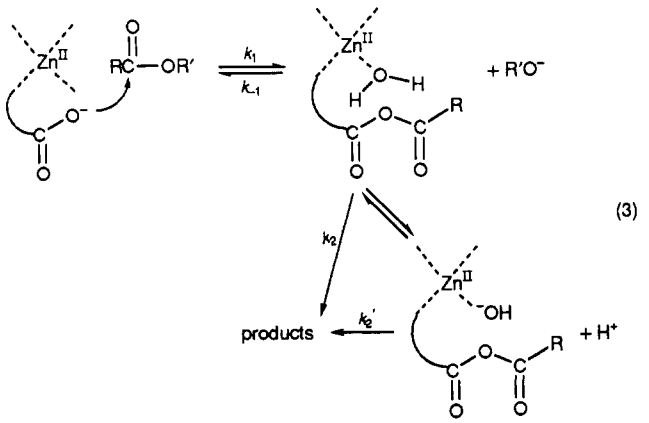
CPA (5.8×10^{-5} M) and **9** (8.7×10^{-5} M or 3.7×10^{-4} M) were incubated at pH 7.5 and 8.8 for several seconds at 0 °C. Precooled guanidine hydrochloride (5.6 M final concentration) was then added. The solution was allowed to stand for 5 min at 0 °C. Acetonitrile was then added to 33%, but **8** was not detected. Another denaturation experiment was carried out with trichloroacetic acid under the same conditions. Trichloroacetic acid was added to give an 8% final concentration. Chloroform was added to extract any **8** from the cloudy solution, but none was found. Both methods were checked by adding **8** to the appropriate solutions. **8** is stable under these conditions and can be easily detected.

The CPA-catalyzed hydrolysis of **9** was examined at pH 7.5 in the presence of 0.1, 0.2, and 0.3 M sodium fluoride, with the ionic strength adjusted to 0.5 M with NaCl. Only small increases in the initial rates were found (34% with 0.3 M NaF).

DISCUSSION

Makinen et al. (1976) have presented evidence for an intermediate in the CPA-catalyzed hydrolysis of *O*-(*trans*-*p*-chlorocinnamoyl)-L,β-phenyllactic acid at subzero temperatures and in a mixed aqueous organic solvent system. It has been suggested that the breakdown of an anhydride intermediate is the rate-determining step (Kaiser & Kaiser, 1972; Makinen et al., 1979, Suh et al., 1985). The D_2O solvent isotope effect at pD = 8.05 ($k_{cat}^{H_2O}/k_{cat}^{D_2O} = 2$) gives some support to this interpretation in the hydrolysis of cinnamoyl-L,β-phenyllactate (CPL) (Kaiser & Kaiser, 1972). However, solvent isotope effects are ambiguous with most enzymes (Jencks, 1963). The ascending arm of the k_{cat} vs pH profile for hydrolysis of CPL at pH >9 might be ascribed to a metal ion-promoted OH^- catalyzed breakdown of the anhydride intermediate (King & Fife, 1983). If this is indeed the case, then the pH-independent reaction from pH 8−9 must be due to a water reaction. The observation of pH-independent reactions in the Ni^{2+}, Co^{2+}, and Zn^{2+} promoted hydrolysis of 2-(6-carboxypicolinic) cinnamic anhydride shows that metal ion-promoted water reactions can be competitive with OH^- catalysis in anhydride breakdown (Fife & Przystas, 1983).

The rate constants for hydrolysis of mixed cinnamic acid anhydrides are at least 10^4 greater than those associated with the intramolecular nucleophilic carboxyl group reactions of phthalate monoesters having poor leaving groups comparable in pK_a to β-phenyllactic acid (Fife & Przystas, 1980, 1982, 1983). Therefore, in view of the great lability of anhydrides, the suggestions of rate-determining anhydride breakdown in the CPA-catalyzed hydrolysis of β-phenyllactate esters pose an interpretive problem. There are two possible explanations for rate-determining anhydride hydrolysis. If anhydride formation is a reversible process, i.e., the scheme of eq 3 is



(3)

being followed, then anhydride hydrolysis (k_2) would be rate determining if $k_{-1} \gg k_2$. However, there is no evidence for reversibility in CPA-catalyzed reactions; experiments seeking to demonstrate reversibility have been negative (Hall & Kaiser, 1967). Anhydride hydrolysis could also be rate determining in the enzymatic reactions if the nucleophilic reaction of Glu-270 was significantly faster than the hydrolysis step, i.e., much more favorable than the intramolecular reactions of phthalate monoesters, even though in the latter case the steric fit is excellent.

If an anhydride intermediate is formed in CPA-catalyzed reactions of *O*-(α-benzoylamino)cinnamoyl-L,β-phenyllactate or the corresponding acetamido derivative, then neighboring

benzamido or acetamido group attack would be expected to yield the oxazolinone, which would absorb strongly at 340–370 nm and would be reasonably stable to hydrolysis. Suh et al. (1985) did not detect an oxazolinone at pH 7.5 in the CPA-catalyzed reactions of these esters. Nevertheless, they concluded that an anhydride is formed and that its hydrolysis is rate limiting (k_{cat} = 0.72 s^{-1} at 25 °C with the acetamido ester). Employing structure–reactivity relationships, we have calculated that at pH 7.5 and 30 °C, k_{obsd} for intramolecular acetamido group attack on an anhydride would be 19 s^{-1}, and the half-life would be 0.04 s (Fife et al., 1988). Since oxazolinone formation is OH^- dependent, whereas the enzyme reaction is pH independent at pH >8, an oxazolinone would be detected more readily at pH 8.5–9.5 than at pH 7.5. We have made a thorough search for the oxazolinone **8** that would be produced if an anhydride intermediate is formed in the CPA-catalyzed hydrolysis of **9**. Suh et al. (1985) had suggested that an anhydride builds up in the reaction with this ester substrate, even though they failed to detect any **8**. However, under the conditions described by Suh et al. (1985), the oxazolinone could not have been detected because of its water insolubility. We found no evidence for the presence of **8** at pH values as high as 9.5, where its formation would be 100-fold faster than at pH 7.5. Nor is **8** detected when the solubility problem is overcome by the addition of acetonitrile either before or after the reaction. We established in separate experiments that **8** does not react with the enzyme.

Steric restriction of the neighboring benzamido group due to binding to the enzyme (Christianson & Lipscomb, 1987) could hinder cyclization. However, binding is an equilibrium process, and such restriction would not be complete. It is unlikely that a highly favorable intramolecular reaction of a good nucleophile and a very reactive acyl derivative would be completely abolished. Denaturation of the enzyme after reaction with **9** also does not bring about the detection of **8**. The simplest explanation is that an anhydride intermediate is *not* formed.

Various nucleophiles have been utilized, e.g., hydroxylamine or alcohols, in attempts to trap an anhydride intermediate in CPA-catalyzed reactions. These attempts have all been unsuccessful but are ambiguous. An alcohol nucleophile might necessarily bind to the enzyme in the site in which the leaving group is strongly bound (the K_i for β-phenyllactate is 6 × 10^{-5} M) (Hall et al., 1969). However, nucleophiles such as fluoride ion should be able to enter the active site and react rapidly with an anhydride. The hydrolysis of the ensuing carboxyl derivative (an acyl fluoride) would be rapid. Turnover of the enzyme would, therefore, be accelerated with an enhanced k_{cat} if anhydride breakdown is rate limiting. Fluoride ion is a potent nucleophile toward various acyl derivatives (Jencks & Carriuolo, 1960; DiSabato & Jencks, 1961) and anhydrides (Bunton et al., 1963). Effects are large; for example, 0.06 M F^- increases the rate of hydrolysis of succinic anhydride by a factor of 4-fold. A fluoride ion binds in the active site of the Mn(II) CPA more strongly than Cl^-, but has no effect on the hydrolysis of hippuryl-L-phenylalanine with either the Mn(II) or Zn(II) enzyme (Navon et al., 1970). The chloride ion is an inhibitor toward peptides, but the effect is slight toward esters (Williams & Auld, 1986). Likewise, F^- has little effect on k_{cat} in the hydrolysis of *O*-(α-benzoylamino)-cinnamoyl-L,β-phenyllactate (**9**), even at very high concentrations (0.3 M). This would not be anticipated if indeed an anhydride builds up in the reaction.

Evidence cited by Suh et al. (1985) for the buildup of an anhydride intermediate in the hydrolysis of **9** included a low apparent K_m and the pH independence of k_{cat}. A low K_m can also be explained by nonproductive binding, especially in view of a relatively small k_{cat} value. Nonproductive binding effects will cancel in the ratio k_{cat}/K_m, and the value of 2 × 10^6 M^{-1} s^{-1} for **9** at pH 7.5 is closely similar to that of 1.1 × 10^6 M^{-1} s^{-1} for CPL. A possible nonproductive binding mode for **9** might place the benzoylamino group in S_1'. Anomalous binding has been noted previously with a phosphonamidate inhibitor analogous to the peptide substrate Cbz-Gly-Phe (Christianson & Lipscomb, 1988).

4-Substituted Cinnamoyl-L,β-phenyllactate Esters. In Figures 3 and 4, log k_{cat} and log k_{cat}/K_m for hydrolysis of a series of substituted cinnamoyl-L,β-phenyllactate esters at pH 8 are plotted vs σ, the Hammett substituent constant (Hammett, 1940). The plots are linear. Kaiser et al. (1974) also found linear plots vs σ° (Taft, 1960) at pH 7.5 with a smaller range of substituents. The lack of a downward bend in the plots shows convincingly that no change in the rate-determining step is occurring with changing substituent groups. That is also the case at pH 6. The slopes (ρ) are closely similar at these pH values bracketing pK_{app}. Therefore, the downward bend in the log k_{cat} vs pH plots at pK_a^{ES} cannot be attributed to a change in the rate-determining step.

The assignment of pK_a^{ES} to ionization of the γ-carboxyl group of Glu-270 is subject to the difficulties outlined in the introduction. An alternative interpretation of the apparent pK_a^{ES} is that it does not represent the ionization of a specific group, but is a composite constant. If an equilibrium step follows the ionization and preceeds the rate-determining step, and if $K_{eq} \gg 1$, then $pK_{app} = p(K_aK_{eq})$, and pK_{app} will be less than pK_a (Bruice & Schmir, 1959). This possibility was discussed in detail in King and Fife (1983). Such an equilibrium step could be the formation of an intermediate, e.g., an anhydride or tetrahedral species resulting from nucleophilic attack at the ester carbonyl, or it could be a simple conformational change of ES. Measurements of the pK_a of the γ-carboxyl group of Glu-270 have given values near 7 (Petra, 1971; Petra & Neurath, 1971) and 6 (Spratt et al., 1983). It can be seen in Table 2 that pK_a^{ES} for **1**–**7** varies from 6.3 to 6.8. In contrast, pK_a^E values determined from plots of log k_{cat}/K_m vs pH are constant at 6.4 and 9.0.[3] Substitution in the substrate should have little or no direct influence on the pK_a of Glu-270 in the ES complex, and the deviation in the pK_{app} values for **2**–**7** is not large. The average pK_a^{ES} for **2**–**7** is 6.6 ± 0.14, which is reasonably close to the pK_1^E and to the reported pK_a values of Glu-270. Therefore, there is no compelling evidence for the buildup of an intermediate in any of these reactions.

The K_m values for **2**–**7** are pH independent from pH 5 to 8, i.e., in the pH range in which k_{cat} is declining with decreasing pH. Thus, the proton can be considered to be a noncompetitive inhibitor in that pH range. This indicates that $K_m = K_s$. Consequently, any equilibrium step or steps following the ionization and prior to the rate-limiting step

[3] The apparent pK_a^E of 9 may be that of metal ion-bound water (Auld et al., 1992), but for alternative views see King and Fife (1983) and references cited therein. The pK_a^E could also be that of a phenolic OH.

cannot have an equilibrium constant or combination of constants greater than unity since K_m could not then equal K_s. Therefore, the small divergence in the pK_a^{ES} values in the series **2**−**7** must be due to environmental and steric effects. The apparent pK_a^{ES} must primarily reflect an ionization.

An intermediate is, of course, not ruled out by these data, only its buildup. Evidence has been previously presented for a pH-dependent conformational change near pH 7 (King et al., 1987). A conformational change occurring near pH 7 would be consistent with the evidence of Galdes et al. (1983) and Geoghegan et al. (1983) for two intermediates before the rate-determining step ($ES_1 \rightleftharpoons ES_2$) in CPA-catalyzed hydrolysis of both esters and peptides.[4]

The slope of the plot of log k_{cat} vs σ in Figure 3 (ϱ) is 0.5. This is more positive than the ϱ value for pH-independent hydrolysis of the 1:1 Zn(II) complex of the mixed anhydrides of substituted cinnamic acids and 6-carboxypicolinic acid; there is little effect of electron withdrawal by the 4-substituent in those reactions ($\varrho \leq 0.1$ at 30 °C). The complexed metal ion provides a 100−200-fold enhancement in the rate of the pH-independent hydrolysis reactions but does not alter the ϱ value. These reactions clearly show the lack of effect of electron withdrawal by substituent groups on metal ion-promoted anhydride hydrolysis. The ϱ near zero very likely reflects a transition state resembling reactants, with very little bond making by the nucleophile. In the CPA-catalyzed reaction, the ϱ for k_{cat} (0.5) is in reasonable accord with rate-determining hydrolysis of a Zn(II) complexed anhydride intermediate only if such an intermediate is at low steady-state concentration ($k_{-1} > k_2$ in eq 3 and $k_{cat}(\text{lim}) = k_1k_2/k_{-1}$). If nucleophilic attack on the ester substrate by the weakly basic carboxylate anion of Glu-270 (k_1) were rate-determining, then the reaction should be influenced by electron withdrawal in the substrate to a greater extent than in the OH^--catalyzed reaction, contrary to observation.

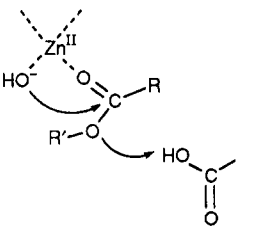
Galdes et al. (1986) suggested that the rate-determining step in the CPA-catalyzed hydrolysis of depsipeptide esters is the release of the product. Teplyakov et al. (1993) proposed that the product complex E·OPhe is stabilized by a hydrogen bond from Zn(II)-bound water whereas the E·Phe complex is not, thereby explaining why product release is rate-limiting for depsipeptide esters but not peptides. For the measured ϱ value for k_{cat} in the reactions of **1**−**7** to be in accord with rate-determining release of the product β-phenyllactate, the magnitude of ϱ would again require a contribution from substituent-dependent preequilibrium steps since otherwise ϱ would be zero.

Clearly, a general scheme involving an anhydride intermediate for the CPA-catalyzed hydrolysis of all ester substrates cannot be achieved, and the evidence for such an intermediate is not conclusive with any substrate. Other types of mechanisms should therefore be assessed, in particular, metal ion-promoted OH^- catalysis, the preferred mechanism in the nonenzymatic hydrolysis of esters (Fife & Przystas, 1982; Fife, 1991). This is the only mechanism known to be capable of generating rate enhancements of the magnitude encountered in CPA-catalyzed reactions.

[4] Conformational effects in CPA-catalyzed reactions will be discussed in detail in a future publication.

The linear plot of log k_{cat}/K_m vs σ (Figure 4) has a slope of 0.92. k_{cat}/K_m is the second-order rate constant for the reaction of substrate with the free enzyme. It is the constant of choice in relative rate studies because it is not affected by any nonproductive binding effects. The ϱ of 0.9 from Figure 4 is almost identical with that for nonenzymatic hydroxide ion-catalyzed hydrolysis of these esters ($\varrho = 0.93$). A plot of log k_{cat}/K_m for the enzymatic reaction at pH 8.0 vs log k_{OH} for alkaline hydrolysis is linear with a slope of 0.9. Thus, electron withdrawal in the acyl group of the ester substrates affects the second-order rate constants for the enzyme reaction and the OH^- reaction alike.

The ϱ values in the enzyme reaction are consistent with a nucleophilic attack in that electron withdrawal facilitates the reaction; ϱ for carboxylate attack on substituted phenolic esters is > 2 (Gaetjens & Morawetz, 1960; Bruice & Benkovic, 1966). The ϱ value for k_{cat}/K_m is that expected for the rate-determining attack of a nucleophile of high basicity, e.g., OH^-, on the ester, and this is supported by the linear correlation of log k_{cat}/K_m and log k_{OH} with a slope of 0.9. The consistent mechanism would involve nucleophilic attack by Zn(II)-bound OH^- as in **10**, perhaps assisted



10

by proton transfer from un-ionized Glu-270. The plot of log k_{cat} vs pH would then be pH independent below the pK_a of metal ion-bound water and would show an inflection influenced by the pK_a of Glu-270 [see the discussion in Fife (1991)]. This reaction could be stepwise or concerted. Note that electron withdrawal in the acyl group would facilitate nucleophilic attack but would hinder proton transfer. The kinetically equivalent mechanism involving proton abstraction from Zn(II)-bound water by the Glu-270 carboxylate anion (Christianson & Lipscomb, 1989) is quite unlikely in view of the low pK_a of the bound water molecule (9 or less) and the known susceptibility of esters to attack by metal ion-bound OH^- (Fife & Pryzstas, 1982). The reaction at pH >6 would proceed entirely through the ionized species, even if its concentration was low (Fife, 1991).

The anhydride mechanism for CPA-catalyzed ester hydrolysis has had a long history and has received considerable support. It has led to much literature discussion and experimental work and may in fact be correct with certain types of ester substrates, e.g., the 4-substituted cinnamoyl-L,β-phenyllactates. However, uncertainties still exist. In the present work, two possible explanations of the ambiguous nature of pK_{app}^{ES}, within the framework of the anhydride mechanism (King & Fife, 1983), have been shown not to be valid. Clearly, serious consideration should be given to other mechanistic schemes and to the possibility that different mechanisms may be utilized depending on the structure of the ester substrate and the manner of its binding in the active site.

REFERENCES

Auld, D. S., & Vallee, B. L. (1970) *Biochemistry 9*, 4352.
Auld, D. S., & Vallee, B. L. (1971) *Biochemistry 10*, 2892.
Auld, D. S., & Holmquist, B. (1974) *Biochemistry 13*, 4355.
Auld, D. S., Bertini, I., Donaire, A., Messori, L., & Moratal, J. M. (1992) *Biochemistry 31*, 3840.
Britt, B. M., & Peticolas, W. L. (1992) *J. Am. Chem. Soc. 114*, 5295.
Bruice, T. C., & Schmir, G. L. (1959) *J. Am. Chem. Soc. 81*, 4552.
Bruice, T. C., & Benkovic, S. (1966) *Bioorganic Mechanisms*, W. A. Benjamin, New York.
Bunting, J. W., Murphy, J., Myers, C. D., & Cross, G. G. (1974) *Can. J. Chem. 52*, 2648.
Bunton, C. A., Fendler, J. H., Fuller, N. A., Perry, S., & Rocek, J. (1963) *J. Chem. Soc.*, 5361.
Carson, F. W., & Kaiser, E. T. (1966) *J. Am. Chem. Soc. 88*, 1212.
Christianson, D. W., & Lipscomb, W. N. (1987) *J. Am. Chem. Soc. 109*, 5536.
Christianson, D. W., & Lipscomb, W. N. (1988) *J. Am. Chem. Soc. 110*, 5560.
Christianson, D. W., & Lipscomb, W. N. (1989) *Acc. Chem. Res. 22*, 62.
de Jersey, J., Kortt, A. A., & Zerner, B. (1966) *Biochem. Biophys. Res. Commun. 23*, 745.
DiSabato, G., & Jencks, W. P. (1961) *J. Am. Chem. Soc. 83*, 4393.
Fife, T. H. (1991) in *Perspectives on Bioinorganic Chemistry*, (Hay, R. W., Ed.) Vol. 1, pp 43−93, JAI Press, London.
Fife, T. H., & Przystas, T. J. (1980) *J. Am. Chem. Soc. 102*, 7297.
Fife, T. H., & Przystas, T. J. (1982) *J. Am. Chem. Soc. 104*, 2251.
Fife, T. H., & Przystas, T. J. (1983) *J. Am. Chem. Soc. 105*, 1638.
Fife, T. H., Przystas, T. J., & Pujari, M. P. (1988) *J. Am. Chem. Soc. 110*, 8157.
Fugita, T., Iwasa, J., & Hansch, C. (1964) *J. Am. Chem. Soc. 86*, 5175.
Gaetjens, E., & Morawetz, H. (1960) *J. Am. Chem. Soc. 82*, 5328.
Galdes, A., Auld, D. S., & Vallee, B. L. (1983) *Biochemistry 22*, 1888.
Galdes, A., Auld, D. S., & Vallee, B. L. (1986) *Biochemistry 25*, 646.
Geoghegan, K. F., Galdes, A., Martinelli, R. A., Holmquist, B., Auld, D. S., & Vallee, B. L. (1983) *Biochemistry 22*, 2255.
Gillespie, H. B., & Snyder, H. R. (1943) *Organic Synthesis*, Collect. Vol. II, p 489, Wiley, New York.
Hall, P. L., & Kaiser, E. T. (1967) *Biochem. Biophys. Res. Commun. 29*, 205.
Hall, P. L., Kaiser, B. L., & Kaiser, E. T. (1969) *J. Am. Chem. Soc. 91*, 485.
Hammett, L. P. (1940) *Physical Organic Chemistry*, Chapter 7, McGraw-Hill, New York.
Hartsuck, J. A., & Lipscomb, W. N. (1971) in *The Enzymes* (Boyer, P., Ed.) 3rd ed., Vol. 3, pp 1−56, Academic Press, New York.
Hass, G. M., & Neurath, H. (1971) *Biochemistry 10*, 3535.
Jencks, W. P. (1963) *Annu. Rev. Biochem. 32*, 657.
Jencks, W. P., & Carriuolo, J. (1960) *J. Am. Chem. Soc. 82*, 1778.
Kaiser, E. T., & Kaiser, B. L. (1972) *Acc. Chem. Res. 5*, 219.
Kaiser, E. T., Chan, T., & Suh, J. (1974) *Adv. Exp. Med. Biol. 48*, 59.
King, S. W., & Fife, T. H. (1983) *Biochemistry 22*, 3603.
King, S. W., Lum, V. R., & Fife, T. H. (1987) *Biochemistry 26*, 2294.
Lipscomb, W. N. (1970) *Acc. Chem. Res. 3*, 81.
Ludwig, M. L., & Lipscomb, W. N. (1973) in *Inorganic Biochemistry* (Eichhorn, G. L., Ed.) p 438, Vol. 1, American Elsevier, New York.
Makinen, M. W., Yamamura, K., & Kaiser, E. T. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3882.
Makinen, M. W., Kuo, L. C., Dymowski, J. J., & Jaffer, S. (1979) *J. Biol. Chem. 254*, 356.
Navon, G., Shulman, R. G., Wyluda, B. J., & Yamane, T. (1970) *J. Mol. Biol. 51*, 15.
Petra, P. H. (1971) *Biochemistry 10*, 3163.
Petra, P. H., & Neurath, H. (1971) *Biochemistry 10*, 3171.
Sander, M. E., & Witzel, H. (1985) *Biochem. Biophys. Res. Commun. 132*, 681.
Simpson, R. T., Riordan, J. F., & Vallee, B. L. (1963) *Biochemistry 2*, 616.
Spratt, T. E., Sugimoto, T., & Kaiser, E. T. (1983) *J. Am. Chem. Soc. 105*, 3679.
Suh, J., & Kaiser, E. T. (1976) *J. Am. Chem. Soc. 98*, 1940.
Suh, J., Cho, W., & Chung, S. (1985) *J. Am. Chem. Soc. 107*, 4530.
Suh, J., Hong, S. B., & Chung, S. (1986) *J. Biol. Chem. 261*, 7112.
Taft, R. W. (1960) *J. Phys. Chem. 64*, 1805.
Teplyakov, A., Wilson, K. S., Orioli, P., & Mangani, S. (1993) *Acta Crystallogr. D49*, 534.
Tomalin, G., Kaiser, B. L., & Kaiser, E. T. (1970) *J. Am. Chem. Soc. 92*, 6046.
Williams, A. C., & Auld, D. S. (1986) *Biochemistry 25*, 94.