82707-37-7; (*E*,*Z*)-**6**, 23017-93-8; (*E*,*E*)-**6**, 63121-48-2; (*E*,*E*)-**7**, 82707-38-8; (*E*,*E*)-**8**, 63121-49-3; (*E*,*Z*)-**9**, 82707-39-9; (*E*,*Z*)-**10**, 5502-91-0; (*E*,*Z*,*Z*,*Z*)-5-HPETE, 70968-82-0; (*E*,*Z*,*Z*,*Z*)-8-HPETE, 70968-80-8; (*E*,*Z*,*Z*,*Z*)-9-HPETE, 70968-79-5; (ALL, *Z*)-10-HPETE, 82707-40-2; (*E*,*Z*,*Z*,*Z*)-11-HPETE, 70968-78-4; (*E*,*Z*,*Z*,*Z*)-15-HPETE, 69371-38-6; (*E*,*Z*,*Z*,*Z*)-12-HPETE, 71030-35-8; 7-DHC, 434-16-2; cholesterol, 57-88-5; α-tocopherol, 59-02-9; α-tocopherolquinone, 7559-04-8.

Carboxypeptidase A Catalyzed Hydrolysis of Thiopeptide and Thionester Analogues of Specific Substrates. An Effect on k_{cat} for Peptide, but Not Ester, Substrates

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Abstract: Carboxypeptidase A (peptidyl-L-amino-acid hydrolase, EC 3.4.17.1) catalyzes the hydrolysis of *N*-(*N*-hippuryl-thioglycyl)-3-phenyl-L-alanine, *O*-(*N*-hippurylglycyl)-3-phenyl-L-lactic acid, and *O*-(*N*-hippurylthioglycyl)-3-phenyl-L-lactic acid, respectively the thiopeptide, ester, and thionester analogues of its specific tripeptide substrate *N*-(*N*-hippurylglycyl)-3-phenyl-L-alanine. Both k_{cat} and k_{cat}/K_m are equal for the ester and thionester substrates, reflecting the equal nonenzymic reactivities for these two compounds. However, k_{cat}/K_m for the thiopeptide is only 0.0009 as large as that for its peptide counterpart. This difference, which cannot be due to any inherent reactivity differences between amides and thioamides, lies in k_{cat}, since thiopeptide and peptide bind equally well. The controlling pK_a's of the k_{cat}– and k_{cat}/K_m–pH profiles match those previously observed for specific ester and peptide substrates. Since rotation about the thioamide bond is about 3 kcal mol^{-1} more difficult than rotation about a peptide bond, these data support a mechanism involving rate-determining bond rotation in peptidase, but not esterase, activity.

Carboxypeptidase A (CPA, peptidyl-L-amino-acid hydrolase, EC 3.4.17.1), a zinc-containing digestive protease, catalyzes the hydrolysis of peptide bonds adjacent to the C-terminal residue of a peptide chain.[1-3] X-ray determination of the structure of CPA and several of its complexes[1,4-7] has not sufficed to define clearly its mechanism of action, even when the myriad of kinetic data[2,8-27] on a variety of substrates is considered.

Of particular interest is the divergence of behavior of CPA toward peptide and ester substrates. The following differences have been noted. (1) For a peptide substrate, replacement of the essential zinc by other metals results in a change in k_{cat} while K_m remains unchanged. For an ester substrate, this behavior is reversed: k_{cat} is invariant as K_m changes.[13] (2) Inhibitors such as phenylpropionate, which presumably mimic a hydrophobic side chain of the C-terminal amino acid of a CPA substrate, are competitive against ester substrates, noncompetitive against peptides.[13] (3) The integrity of Tyr-248 is required for peptidase activity, but not for esterase activity.[1,18]

Cleland has attempted to account for all of the available data with a single mechanism, which differs for peptide and ester substrates only in the identity of the rate-determining step.[28] In this mechanism, the first enzyme–substrate complex (collision complex) is "strain free": a salt bridge is formed between the terminal carboxylate of the substrate and Arg-145, but the side chain of the terminal residue does not fill the enzyme's specificity pocket, nor does the carbonyl group adjacent to the scissile bond interact with the metal. Conversion to the precatalytic complex involves formation of these two latter interactions, which provide the energy for a rotation about the scisssile bond, straining it away from its preferred planar conformation into a less stable geometry that more closely resembles the transition state for the nucleophilic attack (eq 1).

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES_1 \underset{k_4}{\overset{k_3}{\rightleftharpoons}} ES_2 \overset{k_5}{\rightarrow} E + P \qquad (1)$$

ES_1, collision complex; ES_2, precatalytic complex

For peptide substrates, rotation about the peptide bond, with its double-bond character, is difficult, so that k_3 is the rate-deter-

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(1) Hartsuck, J. A.; Lipscomb, W. N. *Enzymes, 3rd Ed.* **1971**, *3*, 1–56.
(2) Kaiser, E. T.; Kaiser, B. L. *Acc. Chem. Res.* **1972**, *5*, 219–224.
(3) Lipscomb, W. N. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 3875–3878.
(4) Lipscomb, W. N.; Hartsuck, J. A.; Reeke, G. N.; Quicho, F. A.; Bethge, P. A.; Ludwig, M. L.; Steitz, T. A.; Muirhead, H.; Coppola, J. C. *Brookhaven Symp. Biol.* **1968**, *21*, 24–90.
(5) Lipscomb, W. N. *Tetrahedron* **1975**, *30*, 1725–1732.
(6) Rees, D. C.; Lipscomb, W. N. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 277–280.
(7) Rees, D. C.; Honzatko, R. B.; Lipscomb, W. N. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 3288–3291.
(8) Hall, P. L.; Kaiser, B. L.; Kaiser, E. T. *J. Am. Chem. Soc.* **1969**, *91*, 485–491.
(9) Kaiser, B. L.; Kaiser, E. T. *Proc. Natl. Acad. Sci. U.S.A.* **1969**, *64*, 36–41.
(10) Auld, D. S.; Vallee, B. *Biochemistry* **1970**, *9*, 602–609.
(11) Auld, D. S.; Vallee, B. *Biochemistry* **1970**, *9*, 4352–4359.
(12) Auld, D. S.; Vallee, B. *Biochemistry* **1971**, *10*, 2892–2897.
(13) Auld, D. S.; Holmquist, B. *Biochemistry* **1974**, *13*, 4355–4361.
(14) DeKoch, R. J.; West, D. J.; Cannon, J. C.; Chasteen, N. D. *Biochemistry* **1974**, *13*, 4347–4354.
(15) Bunting, J. W.; Murphy, J. *Can. J. Chem.* **1974**, *52*, 2640–2647.
(16) Nau, H.; Riordan, J. F. *Biochemistry* **1975**, *14*, 5285–5294.
(17) Kang, E. P.; Storm, C. B.; Carson, F. W. *J. Am. Chem. Soc.* **1975**, *97*, 6723–6728.
(18) Suh, J.; Kaiser, E. T. *J. Am. Chem. Soc.* **1976**, *98*, 1940–1947.
(19) Makinen, M. W.; Yamamura, K.; Kaiser, E. T. *Proc. Natl. Acad. Sci. U.S.A.* **1976**, *73*, 3882–3886.
(20) Breslow, R.; Wernick, D. *J. Am. Chem. Soc.* **1976**, *98*, 259–261.
(21) Breslow, R.; Wernick, D. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 1303–1307.
(22) Bunting, J. W.; Chu, S. S.-T. *Biochim. Biophys. Acta* **1978**, *524*, 142–155.
(23) Bunting, J. W.; Kabir, S. H. *Biochim. Biophys. Acta* **1978**, *527*, 98–107.

0002-7863/82/1504-5221$01.25/0

Table I. Product Analyses by Titration with Ellman's Reagent

added to medium[a]	[Ellman's reagent], mM	[thio acid], μM	[CPA], μM	Δabs_{412}	$\Delta\epsilon_{412}$, M^{-1} cm^{-1}	time,[b] min	yield,[c] %
thiols					13 600[d]	very fast	
thiobenzoic acid	2.93	122		1.311	10 740	50	
5	2.93	48.8		1.077	22 000	52	
5	2.93	48.8		1.045	21 500	55	
5	1.95	48.8		1.089	22 300	50	
5	0.99	24.7		0.510	20 700	55	
5 + CPA + 30.7 μM L-Phe	1.00	30.7	2.22	0.650	21 200	60	
30.7 μM 2	1.00			<0.01		*e*	
CPA	1.00		2.22	<0.01		*e*	
CPA	1.00		8.44	<0.01		*e*	
CPA + 30.7 μM 2[f]	1.00		2.22	0.660		60	100
CPA + 67.2 μM 4[g]	1.00		0.017	1.389		60	96

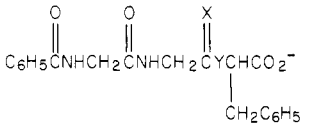
[a] 1 M NaCl, 50 mM Tris, pH 7.50, 1–3 mM Ellman's reagent. [b] Time required for development of maximum absorbance at 412 nm. [c] Of **5**, based on $\Delta\epsilon$ = 21 540, the average value from lines 3–7. [d] Reference 29. [e] No detectable absorbance change after 60 min. [f] Incubated until no further absorbance change at 265 nm was observed. [g] Incubated until no more base was consumed.

mining step, and the Michaelis complex is ES_1. On the other hand, the C–O bond in ester substrates rotates easily, so that ES_2 becomes energetically favored as well as easily formed. The rate-determining step is then one of the succeeding collection of steps represented by k_5, presumably nucleophilic attack by Glu-270 or by water assisted by this residue.

This mechanism makes a simple prediction, namely that any feature of the substrate that reduces the ease of rotation about the scissile bond will result in that substrate being a very poor one for CPA. To test this prediction, we have prepared the thiopeptide and thionester analogues of the specific CPA substrate Bz-Gly-Gly-Phe. We report here determination of the products and rates of CPA-catalyzed hydrolysis of these analogues. A comparison of our results, which show dramatically reduced enzymic activity toward the thiopeptide substrate, to existing data on rotation about the thioamide bond lends strong quantitative support to the Cleland mechanism.

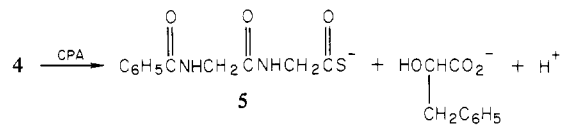
Results

CPA catalyzes the hydrolysis of compounds **2–4**, respectively the thiopeptide, ester, and thionester analogues of its specific tripeptide substrate **1**. In each case, the C-terminal amino or hydroxy acid is released, as expected for CPA-catalyzed hydrolysis.



1, X = O; Y = NH
2, X = S; Y = NH
3, X = O; Y = O
4, X = S; Y = O

Product Analysis. The CPA-catalyzed hydrolysis of ester **3** has been studied previously, but without determination of the products. In this work, prolonged treatment of **3** with CPA consumed 1.0 equiv of base, confirming the expected Bz-Gly-Gly and L-β-phenyllactate as products. Incubation of thionester **4** with CPA



also consumes 1.0 equiv of base, suggesting cleavage at the comparable position to produce the thio acid analague of Bz-Gly-Gly and L-β-phenyllactate. The quantitative uptake of base confirms the stereochemical purity of **3** and **4**, since substrates containing D-amino or -hydroxy acids are known to be resistant to CPA-catalyzed hydrolysis.[1]

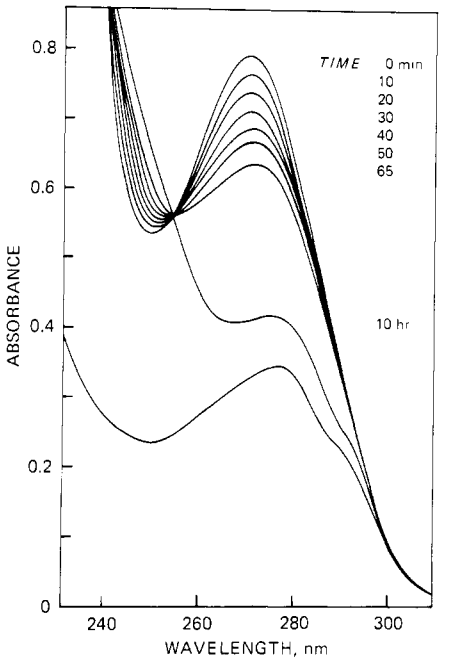


Figure 1. CPA-catalyzed hydryolsis of thiopeptide **2** as monitored by UV spectra. Bottom spectrum: CPA in 1 M NaCl, 50 mM Tris buffer, pH 7.54. Top spectrum: result of addition of **2** to produce concentrations of 41 μM **2**, 2.95 μM CPA. Absorbance decays until the spectrum labeled 10 h is reached. A mixture containing 2.95 μM CPA, 41 μM **5**, and 41 μM 3-phenyl-L-lactic acid in the same buffer produced a spectrum that superimposes on the 10-h spectrum of the reaction mixture.

The identity of thio acid **5** was confirmed by colorimetric assay, based on the sulfhydryl titration by 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent), standardized against **5**, which was prepared by independent synthesis. The solution to be assayed is incubated at room temperature with an excess of Ellman's reagent and the absorbance monitored at 412 nm. Results are shown in Table I. The last line demonstrates the quantitative formation of **5** in the CPA-catalyzed hydrolysis of **4**.

The chemistry of this sulfhydryl titration is not identical with that seen with thiols,[29] since the extinction coefficient of the product(s) differs from that of *p*-nitrothiophenol and also from that of the products formed from thiobenzoic acid. Very likely

(24) Sugimoto, T.; Kaiser, E. T. *J. Am. Chem. Soc.* **1978**, *100*, 7750–7751.
(25) Sugimoto, T.; Kaiser, E. T. *J. Am. Chem. Soc.* **1979**, *101*, 3946–3951.
(26) Makinen, M. W.; Luo, L. C.; Dymowski, J. J.; Jaffer, S. *J. Biol. Chem.* **1979**, *254*, 356–366.
(27) Mock, W. L.; Chen, J.-T. *Arch. Biochem. Biophys.* **1980**, *203*, 542–552.
(28) Cleland, W. W. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1977**, *45*, 273–387.
(29) Ellman, G. L. *Arch. Biochem. Biophys.* **1959**, *82*, 70–77.

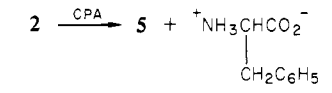
Table II. Controlling pK_a's and Limiting Values (Standard Deviations) of Kinetic Parameters

substrate	k_{cat}, s^{-1}	K_m, μM	k_{cat}/K_m, M^{-1} s^{-1}	pK_{EH_2}	pK_{ESH_2}	pK_{EH}	ref
1	20	1000	20 000				13
1	20	800	25 000				10
1				6.2	6.0	9.0	11
1				6.2		9.1	63
2		500 (110)[a]	22.1 (1.1)			9.2 (0.5)	this work
3	500	330	1.5×10^6				13
3				6.2		9.1	63
3	517 (21)	185 (53)	$1.68\ (0.23) \times 10^6$	5.7 (1.4)	6.4 (0.4)	9.1 (1.3)	this work
4	436 (30)	142 (36)	$1.39\ (0.16) \times 10^6$	6.0 (0.6)	6.7 (1.5)	9.2 (0.6)	this work
4[b]			$0.92\ (0.04) \times 10^6$	6.10 (0.2)		9.5 (0.3)	this work

[a] Determined at pH 7.5 only. [b] First-order kinetic measurements at $[S] \ll K_m$.

the acyl disulfide formed as the primary product undergoes some further reaction. Other differences from the simple Ellman titration include the longer time required for color development and the subsequent fading of the color.

Since the expected CPA-catalyzed cleavage of **2** produces no



protons, another means was devised to confirm the identity of the products. An artificial product mixture containing 1 equiv each of **5** and L-Phe and a catalytic amount of CPA was constructed. Figure 1 shows the indentity of the UV spectrum of this mixture with that produced by prolonged incubation of **2** and CPA. Further, the clean isosbestic point at 254 in Figure 1 shows either **5** is a stable hydrolysis product or if it undergoes further reaction, this reaction is undetectable spectrophotometically.

Verification of the products of CPA-catalyzed hydrolysis of **4** was also attempted by using this technique. The results were inconclusive, since an artificial product mixture with L-β-phenyllactate replacing L-Phe is superimposable on the spectra of both reactants and products of the CPA-catalyzed hydrolysis of **4**. In other words, there is no significant UV change in this reaction since the unconjugated thionester does not provide a strong UV chromophore.

Kinetics. Appearance of primary products was monitored titrimetrically in the case of ester **3** and thionester **4**, whose hydrolyses liberate a proton, and spectrophotometrically in the case of thiopeptide **2**, where destruction of the thioamide chromophore leads to a large decrease in the 260–280-nm region (Figure 1). In all cases, the kinetic measurements are uncomplicated by further reactions of the primary products, should they occur. Although not explicitly ruled out, such further reactions seem unlikely on the basis of first-order kinetics observed with **4**, the isosbestic point in Figure 1, and the known reduced reactivity of CPA toward amides relative to esters.[13]

Initial rates of CPA-catalyzed hydrolysis of **3** and **4**, determined titrimetrically by monitoring the sodium hydroxide uptake required to maintain constant pH, showed good fits to the usual hyperbolic form (eq 2), yielding linear v vs. $v/[S]$ plots. At concentrations

$$v = \frac{k_{cat}E_0[S]}{K_m + [S]} \qquad (2)$$

of **4** well below its K_m, clean first-order kinetics were observed through at least three half-lives. The pseudo-first-order rate constant measured under such conditions, which is simply $k_{cat}E_0/K_m$, agrees reasonably well with the same ratio determined by a series of initial rate measurements. Similar first-order kinetics were not observed in the CPA-catalyzed hydrolysis of **3**. This is consistent with binding of the product dipeptide Bz-Gly-Gly and failure of the corresponding thio acid product, **5**, to inhibit CPA activity significantly.

The pH profiles of the kinetic parameters of **4** are shown in Figures 2 and 3. Nonenzymic hydroxide-catalyzed hydrolysis of both substrates becomes significant above pH 9 and precludes accurate measurement of the enzymic reaction above pH 9.5. Figure 2 shows the pH profile of k_{cat}/K_m for **4** determined by the

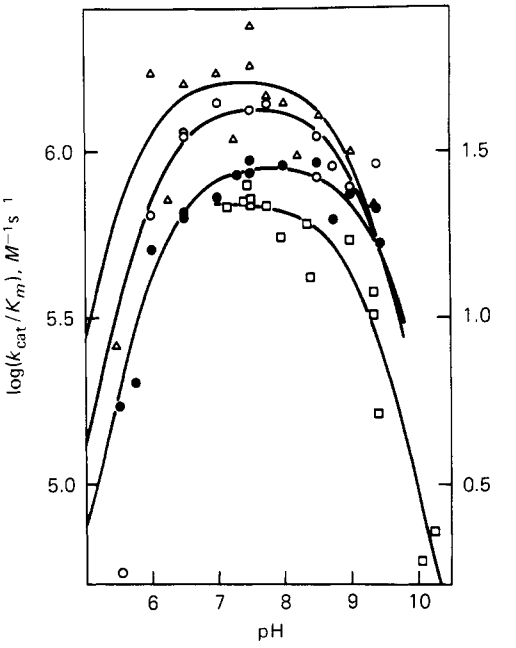


Figure 2. pH profiles of k_{cat}/K_m for the CPA-catalyzed hydrolyses of **2** (□, bottom line, right ordinate scale), **3** (△, top line, left ordinate scale), and **4** (○, second line from top, by initial rates; ●, third line from top, by first-order kinetics, left ordinate scale). Solid lines are calculated for the best fit to eq 3, or in the case of **2** hydrolysis, to eq 5. All runs were carried out at 25 °C in 1.0 M NaCl. Substrate concentrations ranged from 20 to 3000 μM, always spanning K_m, except for first-order kinetic determinations with 12–40 μM **4**. CPA concentrations were 14–18 nM for hydrolyses of **3** and **4**, 4–7 μM for hydrolysis of **2**.

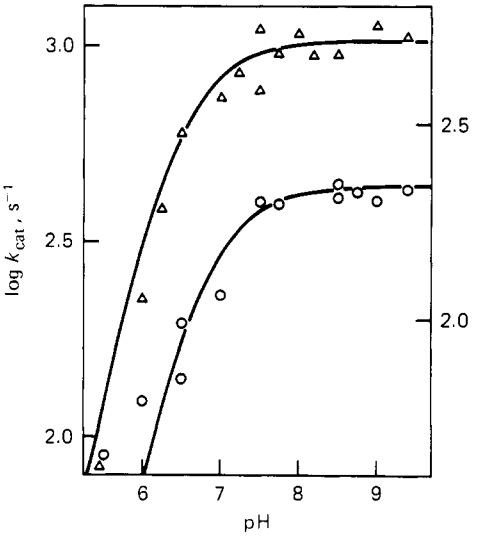


Figure 3. pH profiles of k_{cat} for the CPA-catalyzed hydrolyses of **3** (△, right ordinate scale) and **4** (○, left ordinate scale). Solid lines are calculated for the best fit to eq 4. Experimental conditions are as in Figure 2.

Table III. Effect of Zn^{2+} on CPA-Catalyzed Hydrolysis of 2[a]

added $ZnCl_2$, μM	$10^9 v$, M s^{-1}
none	6.54
4.55	5.18
45.5	0.76
455	<0.001

[a] 25 °C, 1 M NaCl, 50 mM Tris, pH 7.50, 5.84 μM CPA, 111 μM 2.

two different methods. Fitting of the data to the theoretical model for two ionizable groups (eq 3) determines values for pK_{EH_2} and

$$k_{cat}/K_m = \frac{(k_{cat}/K_m)_{lim}}{\frac{[H^+]}{K_{EH_2}} + 1 + \frac{K_{EH}}{[H^+]}} \quad (3)$$

pK_{EH} and for the limiting value of k_{cat}/K_m. These values are collected in Table II.

Unlike k_{cat}/K_m, the kinetic parameter k_{cat} varies in a sigmoid fashion with pH. The pH profile is shown in Figure 3. Fitting to a model with a single ionizable group (eq 4) determines K_{ESH_2}

$$k_{cat} = \frac{k_{cat_{lim}}}{\frac{[H^+]}{K_{ESH_2}} + 1} \quad (4)$$

and $k_{cat_{lim}}$ (Table II). K_{ESH_2} can, in principle, differ from K_{EH_2} for any of several reasons.[28] If binding of substrate is rapid and reversible, a good approximation with CPA,[13] then K_{ESH_2} reflects the pK_a of a group in the enzyme–substrate complex, while K_{EH_2} refers to the free enzyme.[28]

With 3, considerable scatter was obtained at the low substrate concentrations required to obtain k_{cat}/K_m. If the pH profile (Figure 2) is forced to fit a bell-shaped curve (eq 3), values of K_{EH_2}, K_{EH}, and $(k_{cat}/K_m)_{lim}$ can be obtained, albeit with a high uncertainty (Table II). Fewer problems were encountered at saturating substrate concentrations, and the parameter k_{cat} is better behaved, again showing sigmoid behavior (Figure 3). The values of $k_{cat_{lim}}$, $(k_{cat}/K_m)_{lim}$, and the controlling pK_a's are in excellent agreement with those reported in the literature (Table II).

CPA also catalyzes the hydrolysis of the thiopeptide 2. The kinetics of this reaction, which was monitored spectrophotometrically at 265 nm, are complicated both by substrate inhibition and by Zn^{2+} inhibition. Both may be attributed to the known[30-33] affinity of thioamides for zinc ions. In the former case, higher concentrations of substrate pull the zinc from the CPA active site, while in the latter case, the thipeptide–zinc complex has no affinity for the enzyme. Substrate inhibition allows only a rough estimate of K_m from the linear part of a v vs. v/S plot, while k_{cat} cannot be obtained at all. From the abscissa intercept, however, k_{cat}/K_m can be obtained quite satisfactorily. Table III shows the inhibitory effect of added Zn^{2+}.

We were unable to obtain reproducible kinetic data at pH values below 7 in the absence of added Zn^{2+}, an expected consequence of the known[11] requirement of excess Zn^{2+} for CPA stability in this pH region. The result of this combination of inhibition with added zinc and irreproducibility without it is that kinetic data are limited to the pH range above 7. K_{EH} can be determined by fitting the data in the limited pH range (Figure 2) to the equation for a falling sigmoid (eq 5). K_m is estimated from data at the single

$$k_{cat}/K_m = \frac{(k_{cat}/K_m)_{lim}}{1 + \frac{K_{EH}}{[H^+]}} \quad (5)$$

Table IV. Nonenzymic Reactions of Ester/Thionester Pairs

substrate	nucleophile	k, M^{-1} s^{-1}	k_O/k_S	ref
3	OH^-	3.54		
4	OH^-	3.22	1.1	this work
Ac-Phe-OCH_3	OH^-	1.01		
Ac-Thiophe-OCH_3	OH^-	0.82	1.2	this work
$C_6H_5COOC_6H_4NO_2$-*p*	OH^-	1.45		
$C_6H_5CSOC_6H_4NO_2$-*p*	OH^-	0.172	8.4[a]	64
$C_6H_5COOC_6H_4NO_2$-*p*	$CF_3CH_2O^-$	2.13		
$C_6H_5CSOC_6H_4NO_2$-*p*	$CF_3CH_2O^-$	1.63	1.3[a]	64
$C_6H_5COOC_2H_5$	OH^-	0.0280		
$C_6H_5CSOC_2H_5$	OH^-	0.0372	0.75[b]	65
$C_6H_5COO(CH_2)_2N^+(CH_3)_3$	OH^-	0.56		
$C_6H_5CSO(CH_2)_2N^+(CH_3)_3$	OH^-	0.30	1.9[c]	66
$C_6H_5COO(CH_2)_2N(CH_3)_2$	OH^-	0.058		
$C_6H_5CSO(CH_2)_2N(CH_3)_2$	OH^-	0.086	0.67[c]	66

[a] 25 °C, 20% aqueous acetonitrile. [b] 50 °C, 50% aqueous dioxane. [c] 25 °C, water.

pH value of 7.5. The values of the measurable parameters are listed in Table II, along with literature values for the similar peptide substrate 1.

Nonenzymic Reactivities of Thioamides and Thionesters. Extensive study of thioamide hydrolysis[34-39] yields the qualitative conclusion that thioamides are more reactive than their amide analogues. In the best studied case, thioacetamide was shown to be 80 times as reactive as acetamide toward hydroxide[37,38] and equally reactive in acid-catalyzed hydrolysis.[39] Since thionester hydrolysis has been less widely investigated, we have measured the second-order rate constants for the hydroxide-catalyzed hydrolysis of 3, 4, *N*-acetylphenylalanine methyl ester, and the latter thionester analogue. These are reported in Table IV, along with a few values for other ester/thionester pairs from the literature. It is clear that the chemical reactivities of esters and thionester in general, and 3 and 4 in particular, are essentially identical.

The important results may be summarized as follows.

(1) Thionesters and thiopeptides, in which the oxygen of the carbonyl group adjacent to the scissile bond is replaced by sulfur, are hydrolyzed by CPA. That the hydrolysis is a normal enzymic process, following a mechanism parallel to that for ester or peptide hydrolysis, is shown by the facts that (a) the position of cleavage is normal, (b) saturation kinetics are observed, (c) the kinetic parameters are governed by p*K*'s identical with those for specific ester and peptide substrates, and (d) the controlling p*K*'s are unperturbed by substrate binding, behavior parallel to that seen with specific substrates. (2) The thionester 4 is hydrolyzed by CPA with an efficiency, as reflected in k_{cat}/K_m, equal to that observed with its ester counterpart 3, a specific CPA substrate. The enzymic reactivities of this ester/thionester pair parallel their equal chemical reactivities. The strength of binding, as reflected in K_m, is also equal for these two substrates. (3) In contrast, the thiopeptide 2 is hydrolyzed only 0.0009 times as fast as it specific peptide analogue 1. This difference is not due to inherent chemical reactivity, since thioamides are at least as reactive toward hydrolysis as are amides. The difference in efficiency of CPA catalysis resides entirely in k_{cat}, since thiopeptide 2 binds as well as peptide 1.

Discussion

The Cleland mechanism for CPA action[28] postulates rate-determining peptide bond rotation in peptidase activity and rate-

(30) Hall, A. S.; Satchell, D. P. N. *J. Chem. Soc., Chem. Commun.* **1975**, 50–51.
(31) Hall, A. S.; Satchell, D. P. N. *J. Chem. Soc., Perkin Trans. 2* **1975**, 778–784.
(32) Hall, A. S.; Satchell, D. P. N. *J. Chem. Soc., Perkin Trans. 2* **1975**, 1351–1353.
(33) Hall, A. S.; Satchell, D. P. N. *J. Chem. Soc., Perkin Trans. 2* **1975**, 1366–1370.
(34) Hurd, R. N.; DeLaMater, G. *Chem. Rev.* **1961**, *61*, 45–86.
(35) Walter, W.; Voss, J. In "The Chemistry of Amides"; Patai, S., Ed.; Interscience: London, 1970; pp 383–475.
(36) Walter, W.; Voss, J. In "Organic Compounds of Sulphur, Selenium, and Tellurium"; Hogg, D. R., Ed.; Chemical Society: London, 1979; pp 139–186.
(37) Bruylants, A.; Kézdy, F. *Rec. Chem. Prog.* **1960**, *21*, 213–240.
(38) Peeters, O. M.; deRanter, C. J. *J. Chem. Soc., Perkin Trans. 2* **1976**, 1062–1065.
(39) Peeters, O. M.; deRanter, C. J. *J. Chem. Soc., Perkin Trans. 2* **1974**, 1832–1835.

determining covalent bond formation in esterase activity. This leads to the prediction that any difference between rates of enzymic ester and thionester hydrolysis should parallel differences in their chemical reactivities. The results fulfill this prediction exactly, with ester **3** and thionester **4** being equally reactive, both chemically and enzymically. For peptidase activity, however, this mechanism predicts that if the substrate is altered in such a way as to make rotation about the scissile bond more difficult, this effect will be directly reflected in reduced CPA activity, specifically in k_{cat}.

The rotation about the C–N bond in both amides and thioamides has been extensively studied,[40-51] with the conclusion that rotation in thioamides is more difficult. The most systematic study[51] found the simple linear correlation between free energies of activation for rotation

$$\Delta G^{\ddagger}_{\text{thioamide}} = 1.13 + 1.11\Delta G^{\ddagger}_{\text{amide}}$$

where $\Delta G^{\ddagger}$ is measured in units of kcal mol^{-1}. For activation barriers in the 15–20-kcal range, this places the differences in G between amides and thioamides at about 3 kcal mol^{-1}, in agreement with estimates from several different laboratories using ^{1}H[40], ^{13}C[45,47] and ^{15}N[50] NMR. Although the rotational barrier is sensitive to solvent polarity[40] and intramolecular hydrogen bonding,[49] both of which are expected to be significant at the CPA site, we may use the 3 kcal mol^{-1} value as an approximate expected difference in free energies of enzymic transition states, with that involving the thioamide being less favored. The actual ratio between k_{cat}/K_m values is 1100 in favor of the amide **1**, which corresponds to a free-energy difference of 4.1 kcal mol^{-1}, reasonably close to the predicted value. Thus, the Cleland mechanism qualitatively and very nearly quantitatively predicts the reactivity of CPA toward the peptide/thiopeptide pair **1/2** as well as the ester/thionester pair **3/4**. We thus interpret these data as strongly supporting the Cleland mechanism.

Still remaining ambiguous are other aspects of the CPA mechanism on which our data are not so decisive. These include whether Glu-270 acts as a nucleophile[52] or a general base[9] or possibly functions differently for esterase and peptidase activity.[20,21] Since an anhydride intermediate seems established in the esterase mechanism[19,26] and since our data are consistent with a single mechanism for peptidase and esterase activity, we tend to favor the anhydride mechanism, if only for reasons of simplicity.

Also unresolved is the question of whether the substrate carbonyl (or thiocarbonyl) coordinates directly to the Zn, as suggested by X-ray structures of Gly-Tyr[4] and 3-*p*-methoxybenzoyl-2-benzylpropionic acid[7] complexes, or to a Zn-bound water molecule. A Zn-bound water is associated with the low pK_a observed in the pH profile for deacylation of an anhydride intermediate,[26] but this could still be consistent with direct coordination if Zn were to become pentacoordinate.[53] Our data have only an indirect bearing on this question. If deacylation is rate determining for esters,[26] and thus presumably also for thionesters, then comparing rates for these two classes of substrates involves comparison of relative ease of nucleophilic attack by water—either free or Zn bound—on carbonyl vs. thiocarbonyl carbon. Since the nonenzymic reactivities are equal, the identical enzymic rates are not inconsistent with a direct coordination mechanism. Maintaining the idea of direct coordination for esters does force one, however, into the somewhat uncomfortable conclusion that the fit between metal and substrate need not be too precise, since Zn can accommodate sulfur and oxygen equally well. In contrast, the serine proteases, where the electrophilic contact between enzyme and carbonyl heteroatom is known to be direct,[55,56] hydrolyze thionesters much less efficiently than their oxygen analogues.[54]

Finally, we note that this is the first report of the synthesis and properties of a thiopeptide. The fact that it binds to at least one protein in a normal fashion, but strongly resists enzymic degradation, raises the possibility of interesting biological and pharmacological properties of thiopeptide derivatives and biologically active hormones and neurotransmitters. Studies on these lines are currently under way in these laboratories.

Experimental Section

Carboxypeptidase A (Allan, δ, type II), from bovine pancreas, treated with diisopropylphosphofluoridate, was purchased from Sigma Chemical Co. as an aqueous suspension with toluene added. Purified stock solutions were prepared by dissolving 2 mL of aqueous suspension in 5 mL of 2 M sodium chloride solution and eluting this from a 20 × 1.5 cm Sephadex G-25 column with about 15 mL of 2 M sodium chloride solution. Protein concentrations of such solutions were about 40 μM, as determined spectrophotometrically at 278 nm, assuming an extinction coefficient of 1.94 mg mL^{-1} cm^{-1} [57] and a molecular mass of 33 694.5 daltons.[58] Activity was assayed with hippuryl-L-phenylalanine by the method of Folk and Schirmer.[59] For the purposes of calculation of E_0, pure enzyme was taken as that having a specific activity of 38 units mg^{-1}. The specific activity of stock solutions was stable for several days at 4 °C, unlike more dilute solutions (10–100 nM), whose activities decreased sharply within 1 h.

***N*-(*N*-Hippurylthioglycyl)-3-phenyl-L-alanine methyl ester** was synthesized from the corresponding dithioethyl ester[54] and L-phenylalanine methyl ester. To a solution of 510 mg (1.7 mmol) of *N*-hippuryldithioglycine ethyl ester and 1.5 g (6.9 mmol) of L-phenylalanine methyl ester in 10 mL of dimethylformamide in a nitrogen atmosphere was added 300 μL of triethylamine in three portions over a period of 30 h. Thin-layer chromatography (silica gel plates, 30:70 ethanol–chloroform eluant, starting material R_f 0.62, product R_f 0.76) indicated that the reaction was complete in about 40 h at room temperature. Equal amounts, about 10 mL, of 1.5 M HCl and dichloromethane were then added to the reaction mixture. The organic layer was washed with saturated sodium chloride solution, dried over magnesium sulfate, and decolorized with activated carbon. The solvent was evaporated under reduced pressure and the residue crystallized from ethyl acetate–petroleum ether to yield 390 mg (57%): mp 58 °C dec; ^{1}H NMR ($CDCl_3$) δ 3.25 (d, J = 6.5 Hz, 2), 3.63 (s, 3), 4.08 (d, J = 5 Hz, 2), 4.26 (d, J = 5 Hz, 2), 5.35 (m, 1), 7.1–7.9 (m, 12), 8.97 (d, J = 7.5 Hz, 1); ^{13}C NMR ($CDCl_3$) δ 199.7 (C=S), 171.0, 170.0, 168.1 (C=O), 135.8, 133.3, 129.3, 129.1, 128.8, 127.4, 127.3 (aromatic), 59.0 (OCH_3), 52.5 (CH_2–CS), 50.3 (CH), 44.0 (CH_2–CO), 36.5 (CH_2–C_6H_5); IR (KBr) 3400, 1775, 1675 cm^{-1}.

***N*-(*N*-Hippurylthioglycyl)-3-phenyl-L-alanine (2)** was prepared by saponification of the methyl ester. To a solution of 280 mg (0.6 mmol) of *N*-(*N*-hippurylthioglycyl)-3-phenyl-L-alanine methyl ester in 10 mL of dimethylformamide in a nitrogen atmosphere was added 90 mg (2.2 mmol) of sodium hydroxide in 10 mL of water. Thin-layer chromatography (silica gel plates, 30:70 ethanol–chloroform eluant, starting material R_f 0.76, product R_f 0.0) indicated that the reaction was complete in about 2 h at room temperature. An equal volume of chloroform was added to the reaction mixture. The product, which precipitated when the aqueous layer was acidified to pH 1 with concentrated HCl, was twice recrystallized from dimethyl sulfoxide–water to yield 227 mg (84%): mp 183–185 °C; ^{1}H NMR (Me_2SO-d_6) δ 3.25 (d, J = 7 Hz, 2), 3.95 (d, J = 5.5 Hz, 2), 4.18 (d, J = 6 Hz, 2), 5.22 (m, 1), 7.18–8.10 (m, 10), 8.42

(40) Jackman, L. M.; Kavanogh, T. E.; Haddon, R .C. *Org. Magn. Reson.* **1969**, *1*, 109–123.
(41) Stewart, W. E.; Siddal, T. H., III. *Chem. Rev.* **1970**, *70*, 517–551.
(42) Christensen, D. H.; Kortzeborn, R. N.; Bak, B.; Led, J. J. *J. Chem. Phys.* **1970**, *53*, 3912–3922.
(43) Skancke, P. N.; Aanesland, I. *Acta Chem. Scand.* **1972**, *26*, 2614–2622.
(44) Shambhu, M. B.; Digenis, G. A.; Russel, J. *J. Org. Chem.* **1973**, *38*, 1229–1231.
(45) Neuman, C. R.; Jones, V. *J. Org. Chem.* **1974**, *39*, 929–931.
(46) Schauman, E. *Angew. Chem., Int. Ed. Engl.* **1974**, *13*, 350.
(47) Hirsch, J. A.; Augustin, R. L.; Koletav, G.; Wolf, G. H. *J. Org. Chem.* **1975**, *40*, 3547–3550.
(48) Doepke, W.; Bartholme, B.; Gross, T. *Z. Chem.* **1976**, *16*, 327–328.
(49) Berg, U. *Acta Chem. Scand., Ser. B* **19768** *B30*, 695–704.
(50) Martin, G. J.; Dorine, J. P.; Robiller, C. R.; Martin, M. L. *J. Am. Chem. Soc.* **1977**, *99*, 1381–1384.
(51) Leopardi, C. P.; Fabre, O.; Zimmerman, D.; Reisse, J.; Cornea, F.; Fulea, C. *Can. J. Chem.* **1977**, *55*, 2649–2655.
(52) Reeke, G. N.; Hartsuck, J. A.; Ludwig, M. L.; Quicho, F. A.; Steitz, T. A.; Lipscomb, W. N. *Proc. Natl. Acad. Sci. U.S.A.* **1967**, *58*, 2220–2226.
(53) Kuo, L. C.; Makinen, M. W. Conference on Methods for Determining Metal Ion Environments in Proteins, Jan 10–12, 1979, Las Cruces, NM, Abstract 27.
(54) Nashed, N. T. Ph.D. Thesis, New York University, 1981.
(55) Robertus, J. R.; Kraut, J.; Alden, R. A.; Birktoft, J. J. *Biochemistry* **1972**, *11*, 4293–4303.
(56) Steitz, T. A.; Henderson, R.; Blow, D. M. *J. Mol. Biol.* **1969**, *46*, 337–348.
(57) Allan, B. J.; Keller, B. J.; Neurath, H. *Biochemistry* **1964**, *3*, 40–47.
(58) Bradshaw, R. A.; Ericsson, L. H.; Walsh, K. A.; Newrath, H. *Proc. Natl. Acad. Sci. U.S.A.* **1969**, *63*, 1389–1394.
(59) Folk, J. E.; Schirmer, E. W. *J. Biol. Chem.* **1963**, *238*, 3884–3894.

(t, J = 6 Hz, 1), 8.93 (t, J = 5.5 Hz, 1), 9.91 (d, J = 8 Hz, 1); ^{13}C NMR (Me_2SO-d_6) δ 200.5 (C=S), 171.0, 169.2, 167.0 (C=O), 137.0, 133.9, 129.0, 128.2, 128.0, 127.5, 127.4 (aromatic) (the other carbons could not be separated from the solvent signals); IR (KBr) 3400, 3100, 1775, 1675, 1650, 1175 cm^{-1}; UV (water) λ_{max} 265 nm (ϵ_{max} 10 400 M^{-1} cm^{-1}); $[\alpha]^{25}_D$ +50.7° (c 2, DMF). Anal. Calcd for $C_{20}H_{21}N_3O_4S$: C, 60.14; H, 5.30; N, 10.52; S, 8.03. Found: C, 60.04; H, 5.13; N, 10.66; S, 7.66.

O-(N-Hippurylthioglycyl)-3-Phenyl-L-lactic acid (4) was prepared from the corresponding nitrile via the imidate. A solution of 3.0 g (12.7 mmol) of (*N*-benzoylglycyl)amidoacetonitrile and 2.3 g (13.9 mmol) of 3-phenyl-L-lactic acid in 25 mL of dry tetrahydrofuran was saturated with hydrogen chloride at 0 °C and stirred for 2 h at this temperature. The apparatus was thoroughly flushed with nitrogen, and 25 mL of dry pyridine was added slowly, care being taken to keep the solution cold. While maintained in a nitrogen atmosphere, the reaction mixture was allowed to warm to room temperature and then saturated with hydrogen sulfide and stirred for 1 h. The solvent was removed under reduced pressure, and the oily residue solidified on treatment with 2 M HCl. The crude product was washed with chloroform to remove polymeric material and recrystallized from dimethyl sulfoxide–water–acetone, yielding 300 mg (5.9%): mp 198–200 °C dec; ^{1}H NMR (Me_2SO-d_6) δ 3.32 (d, J = 6.5 Hz, 2), 4.00 (d, J = 6 Hz, 2), 4.17 (d, J = 6 Hz, 2), 5.72 (t, J = 6 Hz, 1), 7.30–8.10 (m, 10), 8.30–9.00 (m, 2); ^{13}C NMR (Me_2SO-d_6) δ 218.7 (C=S), 169.2, 169.1, 166.6 (C=O), 136.1, 134.1, 129.1, 128.4, 128.2, 127.5, 126.9 (aromatic) (the other carbons could not be separated from the solvent signal); IR (KBr) 3400, 3100, 1750, 1675, 1650, 1160 cm^{-1}; $[\alpha]^{25}_D$ +11.8° (c 2 DMF). Anal. Calcd for $C_{20}H_{20}N_2O_5S$: C, 59.99; H, 5.03; N, 7.00; S, 8.01. Found: C, 59.88; H, 4.94; N, 7.07; S, 7.92.

O-(N-Hippurylglycyl)-3-phenyl-L-lactic acid (3) has been reported in the literuture but without any synthetic method or physical properties. We prepared it by coupling with carbonyldiimidazole. To a solution of 369 mg (1.6 mmol) of *N*-hippurylglycine in dry pyridine at 0 °C was added 266 mg (1.6 mmol) of carbonyldiimidazole and the solution stirred until effervescence stopped, about 75 min. Then 200 mg (1.6 mmol) of 3-phenyl-L-acetic acid was added and the mixture stirred for 1 h at 0 °C and 48 h at room temperature. The solvent was evaporated under reduced pressure and the residue crystallized from acetone–water. Recrystallization from acetone yielded 400 mg (65%): mp 190–195 °C dec; ^{1}NMR (Me_2-d_6) δ 3.11 (d, J = 5.5 Hz, 2), 3.94 (d, J = 6 Hz, 4), 5.13 (t, J = 5.5 Hz, 1), 7.23–8.03 (m, 10), 8.43 (t, J = 6 Hz, 1); IR (KBr) 3500, 3100, 1750, 1675, 1650 cm^{-1}; $[\alpha]^{25}_D$ −9.69° (c 1, DMF). Anal. Calcd for $C_{20}H_{20}N_2O_6$: C, 62.49; H, 5.24; N, 7.29. Found: C, 62.42; H, 5.27; N, 7.26.

***N*-Hippurylthioglycine** was prepared by the method of Gisin et al.[60] To a solution of 2.0 g (8.5 mmol) of *N*-hippurylglycine in dry pyridine at 0 °C was added 1.5 g (9.0 mmol) of carbonyldiimidazole. When effervescence had stopped, about 90 min, the reaction mixture was saturated with hydrogen sulfide and stirred for 48 h at room temperature. The solvent was evaporated under reduced pressure and the residue dissolved in ethyl acetate. This solution was washed twice with 2 M HCl and once each with water and saturated sodium chloride solution and dried over magnesium sulfate. The product crystallized upon addition of petroleum ether. Recrystallization from acetone–water and from chloroform–pentane yielded 950 mg (44%): mp 144–147 °C dec; ^{1}H NMR (DMF-d_7) δ 4.14 (d, J = 6 Hz, 2), 4.24 (d, J = 6 Hz, 2), 6.90 (br s, 1), 7.30–8.00 (m, 5), 8.15–9.05 (m, 2); ^{13}C NMR (DMF-d_7) δ 171.3, 170.1, 167.2 (C=O), 134.4, 131.5, 128.4, 127.6 (aromatic), 41.2, 43.3 (CH_2); IR (KBr) 3600, 3300, 2460, 1700, 1675, 1650 cm^{-1}. Anal. Calcd for $C_{11}H_{12}N_2O_3S$: C, 52.35; H, 4.79; N, 11.11; S, 12.72. Found: C, 52.56; H, 4.78; N, 10.93; S, 12.70.

***N*-Acetyl-3-phenyl-*dl*-thioalanine *O*-methyl ester** was prepared from 2.5 g of the corresponding nitrile[54] by the method described above for *O*-(*N*-hippurylglycyl)-3-phenyl-L-lactic acid: yield 510 mg (16%); mp 90–91 °C; ^{1}H NMR ($CDCl_3$) δ 1.93 (s, 3), 3.15 (d, J = 7 Hz, 2), 4.03 (s, 3), 5.10 (m, 1), 6.42 (br s, 1), 7.22 (m, 5); ^{13}C NMR ($CDCl_3$) δ 221.2 (C=S), 169.0 (C=O), 136.3, 129.0, 128.1, 127.2 (aromatic), 61.0 (CH_3CO), 58.8 (OCH_3), 41.0 (CH), 23.0 (CH_2). Anal. Calcd for $C_{12}H_{15}NO_2S$: C, 60.73; H, 6.37; N, 5.90; S, 13.51. Found: C, 60.69; H, 6.33; N, 5.83; S, 13.50.

Pyridine, triethylamine, tetrahydrofuran, and benzene were distilled from calcium hydride. Chloroform and dichloromethane were purified by extraction with concentrated sulfuric acid, water, sodium bicarbonate, and saturated sodium chloride sequentially and then dried over anhydrous magnesium sulfate and distilled from calcium hydride. Dimethylformamide and thiobenzoic acid were fractionally vacuum distilled (bp 30 °C (10 torr) and 84–89 °C (15 torr), respectively). Water was twice distilled, the second distillation in glass vessels. Carbonyldiimidazole was recrystallized from dry benzene. All other materials were of the highest commercial grade available and were used without further purification.

^{1}H and ^{13}C nuclear magnetic resonance spectra were recorded on a Hitachi Perkin-Elmer R20 spectrometer at 60 MHz and a Varian XL-100 spectrometer at 25.2 MHz. Infrared and ultraviolet spectra were recorded on Perkin-Elmer 137 and Cary 15 spectrophotometers. Optical rotations were measured on a Perkin-Elmer 141 polarimeter equipped with a 1-mL cell. Melting points were recorded on a Thomas-Hoover melting point apparatus and are reported uncorrected. Elemental analyses were performed by Spang Microanalytical Laboratory, Eagle Harbor, MI.

Kinetic measurements were carried out titrimetrically by using a pH-stat apparatus, consisting of a Radiometer 26 pH meter, ABU12 autoburet, 11 titrator, SBR-2c recorder, TTA-31 titration assembly, and K4112 calomel and G2222c glass electrode, or spectrophotometrically by using a Gilford 240 spectrophotometer. In each case, temperature was maintained at 25 °C with a Lauda K2 constant-temperature circulator. Titrimetric measurements were performed under a nitrogen atmosphere to eliminate base uptake caused by carbon dioxide absorption.

In a typical titrimetric measurement, 1.5 mL of 1 M sodium chloride solution and 10–30 μL of CPA stock solution were added to the titration vessel and adjusted to the desired pH by addition of 2 M sodium hydroxide solution from the autoburet. After temperature equilibration, 200 μL of an aqueous stock solution of the sodium salt of the substrate was added to begin the reaction. Control experiments with enzyme absent showed no or negligibly small uptake of base caused by addition of substrate alone, except at higher pH values, where hydroxide-catalyzed hydrolysis became appreciable. In such cases, the nonenzymic rate was subtracted from the enzymic rate at each substrate concentration. At pH values <7, $ZnCl_2$ was added, typically at a concentration of 5 μM. Control experiments showed that above the threshold required for enzyme stability, the zinc concentration did not affect the rate of hydrolysis of **3** or **4**.

In a typical spectrophotometric measurement, 100–300 μL of CPA stock solution was added to 2 mL of appropriate 50 mM buffer containing 1 M sodium chloride. After temperature equilibration, substrate was added as in titrimetric measurements to begin the reaction. Control experiments with no enzyme showed no absorbance change.

The kinetic parameters k_{cat} and K_m were determined from initial rate measurements of several runs with varying substrate concentrations by using the unweighted nonlinear least-squares method of Wilkinson.[61] The fitting of the pH profile data to eq 3 and 4 and the calculation of uncertainties were carried out by using the iterative least-squares matrix techniques described by Cleland.[62] Determinations of k_{cat}/K_m by first-order kinetics produced plots conforming to exponential decay with correlation coefficients of 0.99 or higher, with rate constants for identical runs agreeing to within 5–8%.

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(60) Gisin, B. F.; Merrifield, R. B.; Tostesen, D. *J. Am. Chem. Soc.* **1969**, *91*, 2691–2695.
(61) Wilkinson, G. N. *Biochem. J.* **1961**, *80*, 324–332.
(62) Cleland, W. W. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1967**, *29*, 1–32.
(63) Auld, D. S.; Holmquist, B. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **1972**, *31*, 435.
(64) Lapinskas, B. A. Ph.D. Thesis, New York University, 1979.
(65) Smith, S. G.; Feldt, R. J. *J. Org. Chem.* **1968**, *33*, 1022–1027.
(66) Bruice, P. Y.; Mautner, H. G. *J. Am. Chem. Soc.* **1973**, *95*, 1582–1586.