Nonspecific Esterase Activity of Carboxypeptidase A. Specificity for the Alcohol Moiety of *p*-Nitrobenzoate Esters

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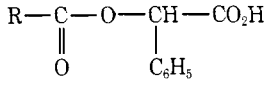
Abstract: A series of 11 *p*-nitrobenzoate esters ($NO_2C_6H_4CO_2CHRCO_2H$ (**2**): R = H, CH_3, CH_3CH_2, $CH_3(CH_2)_2$, $CH_3(CH_2)_3$, $(CH_3)_2CH$, $(CH_3)_2CHCH_2$, C_6H_5, $C_6H_5CH_2$, $C_6H_5(CH_2)_2$, *p*-ClC_6H_4) has been investigated as nonspecific ester substrates of bovine pancreatic carboxypeptidase A at pH 7.5, 25 °C, ionic strength 0.5. The L isomers of all esters except **2** (R = *p*-ClC_6H_4) are hydrolyzed by the enzyme, and k_{cat} and K_m have been evaluated. The results indicate a marked preference in both k_{cat} and K_m for substrates with hydrophobic side chains (R) provided that R does not become too large (e.g., R = $C_6H_5(CH_2)_2$). There is a linear correlation between log K_m (**2**) and log K_i for the competitive inhibitors $RCH_2CO_2^-$: log K_m = 0.61 log K_i − 0.77. Comparison of **2** with the corresponding series of hippurate esters ($C_6H_5CONHCH_2CO_2CHRCO_2H$ (**3**)) indicates that $(k_{cat}/K_m)^2/(k_{cat}/K_m)^3$ is independent of R within experimental error and suggests a common hydrophobic binding region on the enzyme for the R side chain of the alcohol unit of both specific and nonspecific ester substrates. The following species are all competitive inhibitors for the enzymic hydrolysis of **2** (R = $C_6H_5CH_2$): **2** (R = *p*-ClC_6H_4), D-**2** (R = C_6H_5), $C_6H_5CH_2CO_2^-$, $C_6H_5(CH_2)_2CO_2^-$, $C_6H_5CH(CO_2^-)_2$, *p*-$NO_2C_6H_4CO_2^-$, *N*-chloroacetyl-L-phenylalanine. Comparison of the reversible inhibition of the nonspecific esterase, specific esterase, and peptidase activities of carboxypeptidase A allows the construction of a schematic diagram for the binding of these three classes of substrates and related reversible inhibitors. Although the side chains of the alcohol units of specific and nonspecific ester substrates bind in the same hydrophobic region, the terminal carboxylate ions of these two classes of ester substrate are involved in *different* interactions with the enzyme.

A variety of pH dependences has been observed for the hydrolysis of ester and amide substrates by bovine pancreatic carboxypeptidase A.[1-12] There are significant differences in the pH dependences of ester and amide hydrolysis by this enzyme, and furthermore, a variety of pH-rate profiles has been observed for ester substrates.[3,4,7-11] These differences in pH dependence are sufficiently diverse that it has not been possible to accommodate all substrates to a common hypothesis for the mechanism of enzymic hydrolysis. This problem is further exacerbated by the fact that none of the pK_a values which have been found to control substrate binding and hydrolysis has been definitively assigned to any of the ionizable groups which have been shown to be present in the active site of this enzyme.[13]

The binding of all peptide substrates studied to date has been found from the pH dependence of k_{cat}/K_m to be controlled by two ionizable groups on the enzyme with pK_a values ~6 and ~9.[5,6] Similar pK_a values control the binding of depsipeptide ester substrates[7,8,11] and cinnamate ester substrates,[4,10] although the pH dependence of k_{cat} for such ester substrates is different than for peptide substrates. However, a quite different pH dependence for k_{cat}/K_m has been observed for *O*-acetyl-L-mandelic acid (**1**, R = CH_3)[3] and *O*-*p*-nitrobenzoyl-L-



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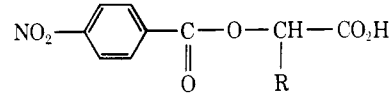
mandelic acid (**1**, R = C_6H_5).[8] Each of these esters displays a bell-shaped pH dependence for k_{cat}/K_m which is consistent with the binding of these two substrates to the enzyme being controlled by enzymic functional groups of pK_a ~6.9 and ~7.7. Thus, there appear to be two quite distinct pH profiles for substrate binding to carboxypeptidase A, and we have suggested[8] that members of this latter class of esters should be referred to as nonspecific ester substrates of this enzyme. (The pH dependence of substrate binding can then conveniently be used to classify substrates as specific and nonspecific substrates.) Such nonspecific substrates are structurally different from the natural peptide substrates and related depsipeptide ester substrates (i.e., specific ester substrates) in that they lack any amide bonds in the acyl moiety of the bond susceptible to hydrolysis. This classification on the basis of structure is similar to that in general use for substrates of the serine proteases, for which amino acid derived substrates are considered to be specific substrates, whereas other esters and amides are designated as nonspecific substrates (e.g., *p*-nitrophenyl acetate).[14]

The quite different pH profiles for the hydrolysis of specific and nonspecific substrates by carboxypeptidase A are suggestive of significant differences in the mechanisms of hydrolysis of these two classes of substrates. It should also be noted that evidence has been steadily accumulating recently for quite different catalytically productive binding sites on this enzyme for specific ester and peptide substrates.[15-18]

In order to augment the meager data that are currently available on the details of the nonspecific esterase activity of carboxypeptidase A, we now wish to report a study of the hydrolysis of a series of *p*-nitrobenzoate esters (**2**) by this enzyme.



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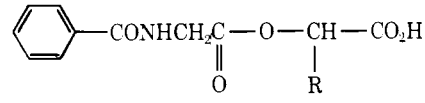
By comparing the enzymic specificity for the R side chain of the alcohol unit of these nonspecific ester substrates with the enzymic specificity for the corresponding substituent in the specific hippurate ester substrates (**3**) we also hoped to gain



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further insight into the relationship between the binding of these two classes of substrates to the enzyme. We have also compared the reversible inhibition of the enzymic hydrolysis of these two classes of substrates by a variety of carboxylate ion inhibitors.

Experimental Section

Synthesis of Substrates. All *p*-nitrobenzoate esters (**2**) were synthesized by the following general route. Pyridine (0.06 mol) was added dropwise to a stirred mixture of *p*-nitrobenzoyl chloride (0.031 mol) and the appropriate 2-hydroxycarboxylic acid[19] (0.03 mol) in anhy-

Table I. *p*-Nitrobenzoate Esters (**2**)

R	Isomer	Mp, °C	^{1}H NMR Spectrum (CD_3COCD_3), δ	Analysis
H[a]		140	4.95 (3 H, s), 3.28 (4 H, s)	($C_9H_7NO_6$) C, H, N
CH_3	L	137	1.7 (3 H, d), 5.42 (1 H, q), 8.45 (4 H, s)	($C_{10}H_9NO_6$) C, H, N
$CH_3(CH_2)_2$	DL	115	1.1 (3 H, t), 1.40–2.25 (4 H, m), 5.40 (1 H, t), 8.45 (4, H, s)	($C_{12}H_{13}NO_6$) C, H, N
$CH_3(CH_2)_3$	DL	102	1.05 (3 H, t), 1.3–1.85 (6 H, m), 5.40 (1 H, t), 8.45 (4 H, s)	($C_{13}H_{15}NO_6$) C, H, N
$(CH_3)_2CH$	DL	94	1.25 (6 H, d), 2.20–2.60 (1, H, m), 5.25 (1 H, d), 8.45 (4 H, s)	($C_{12}H_{13}NO_6$) C, H, N
$(CH_3)_2CH$-CH_2	DL	115	1.02 (6 H, d), 1.65–2.20 (3 H, m), 5.35 (1 H, t), 8.45 (4 H, s)	($C_{13}H_{15}NO_6$) C, H, N
$CH_3(CH_2)_5$	DL	120	0.95 (3 H, t), 1.10–2.00 (10 H, m), 5.35 (1 H, t), 8.40 (4 H, s)	($C_{15}H_{19}NO_6$) C, H, N
C_6H_5	D	140	6.30 (1 H, s), 7.30–7.90 (5 H, m), 8.45 (4 H, s)	($C_{15}H_{11}NO_6$) C, H, N
C_6H_5	DL	174	6.30 (1 H, s), 7.30–7.90 (5 H, m), 8.45 (4 H, s)	($C_{15}H_{11}NO_6$) C, H, N
$C_6H_5CH_2$	L	108	3.40 (2 H, m), 5.55 (1 H, m), 7.30 (5 H, m), 8.40 (4 H, s)	($C_{16}H_{13}NO_6$) H, N
$C_6H_5CH_2$	DL	161	3.40 (2 H, m), 5.55 (1 H, m), 7.30 (5 H, m), 8.40 (4 H, s)	($C_{16}H_{13}NO_6$) C, H, N
$C_6H_5(CH_2)_2$	DL	117	2.45 (2 H, t), 2.85 (2 H, q), 5.35 (1 H, t), 7.30 (5 H, s) 8.35 (4 H, s)	($C_{17}H_{15}NO_6$) C, H, N
p-ClC_6H_4	DL	119	6.35 (1 H, s), 7.40–7.80 (4 H, m), 8.45 (4 H, s)	($C_{15}H_{10}ClNO_6$) C, H, N

[a] Synthesized by Dr. J. Murphy.

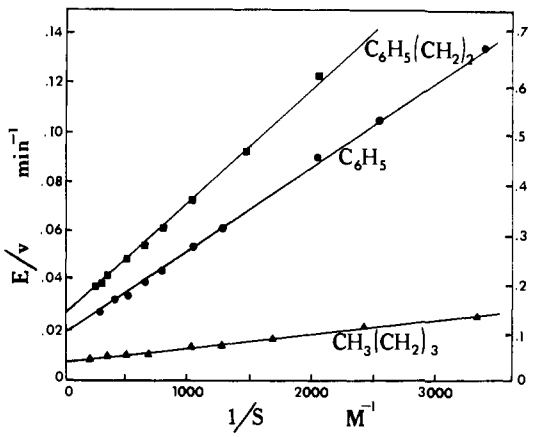

Figure 1. Lineweaver-Burk plots for the enzymic hydrolysis of selected *p*-nitrobenzoate esters (**2**). R side chains are indicated on each line: R = $CH_3(CH_2)_3$, C_6H_5 (left-hand ordinate); R = $C_6H_5(CH_2)_2$ (right-hand ordinate).

Table II. Hydrolysis of *p*-Nitrobenzoate Esters (**2**) by Carboxypeptidase A[a]

R	k_{cat}, min^{-1}	10^4K_m, M	$10^{-5}k_{cat}/K_m$, M^{-1} min^{-1}
H[b]	2.2	250	0.00088
CH_3	55	94	0.058
C_2H_5[c]	110	25	0.44
$CH_3(CH_2)_2$	200	11	1.8
$CH_3(CH_2)_3$	130	7.1	1.8
$(CH_3)_2CH$	58	9.1	0.64
$(CH_3)_2CHCH_2$	120	11	1.1
C_6H_5	50	15	0.33
$C_6H_5CH_2$	210	3.1	6.8
$C_6H_5(CH_2)_2$	7.2	16	0.045

[a] At pH 7.5, 25 °C, ionic strength 0.5. All data based on concentration of L isomer of racemic mixtures. Experimental errors in k_{cat} and K_m are in the range ±2–6%. [b] From ref 27. [c] From ref 22.

drous tetrahydrofuran (50 mL). The resultant mixture was stirred at room temperature for 18 h. Pyridine hydrochloride was filtered off and the tetrahydrofuran removed under vacuum to give a viscous oily residue. The residue was dissolved in dichloromethane (200 mL), and this solution was extracted with ice-cold 3 M hydrochloric acid (35 mL). After drying the organic layer over anhydrous magnesium sulfate, the dichloromethane was removed under vacuum to give the crude ester. This crude product was repeatedly recrystallized from benzene until pure. Repeated crystallization was necessary to remove *p*-nitrobenzoic acid, and the final yields of purified product were quite low (10–20%). The melting points and ^{1}H NMR spectra of esters synthesized in this way are collected in Table I.

Substrate Stock Solutions. Stock solutions (0.01 M) of each ester were prepared as follows.[21] The ester was dissolved in acetone, and exactly 1 equiv of aqueous base was added to generate the carboxylate anion of the ester. Acetone was completely removed on the rotary evaporator, and sufficient sodium chloride was added to give a final ionic strength of 0.5. This solution was made up to near the desired volume with water and the pH carefully adjusted to 7.5. Further water was then added to give exactly the desired volume.

Enzyme Stock Solutions. Stock solutions of carboxypeptidase A were prepared as previously described[22] by dialysis of the toluene-preserved suspension of the enzyme (Code CoA) prepared by Worthington Biochemical Corp.

Kinetic Studies. The enzymic hydrolysis of each ester was followed at a series of substrate concentrations on a Radiometer Corp. pH-stat at pH 7.5, 25 °C, ionic strength 0.5, using standard 0.01 M KOH solution as titrant. Initial velocities at each substrate concentration were calculated from the recorded curves of volume of titrant against time. The data were plotted in the form of Lineweaver-Burk plots (e.g., Figure 1), and the linear regions of such plots were fitted to the Michaelis equation by a computer program based on the nonlinear regression technique of Wilkinson.[23] Reversible inhibition studies were carried out as previously described[18,24-26] at pH 7.5, 25 °C, ionic strength 0.2. Over the complete range of work reported herein, enzyme concentrations varied in the range 1×10^{-8}–2×10^{-6} M.

Results

Lineweaver-Burk plots for the hydrolysis of several *p*-nitrobenzoate esters by carboxypeptidase A are shown in Figure 1 at pH 7.5, 25 °C, and ionic strength 0.5 (NaCl). For all esters investigated, such plots are linear over a considerable range of substrate concentrations spanning the K_m value, although in several cases substrate inhibition is apparent at the highest substrate concentrations investigated. Values of k_{cal} and K_m obtained for all *p*-nitrobenzoate esters amenable to investigation in the present work are collected in Table II, and this table also includes data on two other *p*-nitrobenzoate ester substrates that have been studied previously.

In all cases where racemic esters were used as substrates, only 50% of the ester was enzymically hydrolyzed at pH 7.5. Kinetic parameters obtained from the L and DL isomers of *O*-*p*-nitrobenzoyl-3-phenyllactic acid (**2**, R = $C_6H_5CH_2$) agree within experimental error: k_{cal}(L) = 250 min^{-1}, k_{cat}(DL) = 270 min^{-1} and K_m(L) = 2.9×10^{-4} M, K_m(DL) = 2.8×10^{-4} M (at ionic strength 0.2, assuming only the L ester binds). Thus, for this ester only the L isomer is a substrate and the D isomer is neither a substrate nor inhibitor over the range of the linear Lineweaver-Burk plots. To further check this point, *O*-*p*-nitrobenzoyl-D-mandelic acid (**2**, R = C_6H_5) was syn-

Table III. Reversible Inhibition of Carboxypeptidase A Hydrolyses[a]

Inhibitor	Substrate		
	2, R = $C_6H_5CH_2$	**3**, R = $C_6H_5CH_2$	Peptides
$C_6H_5CH_2CO_2^-$	Comp (K_i = 1.1 mM)	Comp (K_i = 0.52 mM)[b]	Noncomp (K_i = 0.73 mM)[f]
$C_6H_5(CH_2)_2CO_2^-$	Comp (K_i = 0.07 mM)	Comp (K_i = 0.17 mM)[b]	Noncomp (K_i = 0.12 mM)[f]
$C_6H_5CH(CO_2^-)_2$	Comp (K_i = 0.25 mM)	Comp (K_i = 0.16 mM)[c]	Comp[g]
p-$NO_2C_6H_4CO_2^-$	Comp (K_i = 28 mM)	Noncomp (K_i = 22 mM)[d]	
N-$ClCH_2CONHCH(CH_2C_6H_5)CO_2^-$	Comp (K_i = 1.6 mM)	Noncomp (K_i = 0.51 mM)[e]	(Comp)[h]

[a] Data for ester substrates at pH 7.5, 25 °C, ionic strength 0.2. [b] From ref 24. [c] From ref 25. [d] From ref 26. [e] From ref 18. [f] From ref 28. [g] Phenylmalonate dianion has not been examined as an inhibitor of peptidase activity. However, benzylmalonic acid and other malonic acid derivatives are competitive inhibitors.[29] [h] *N*-Chloroacetyl-L-phenylalanine appears to be a typical peptide substrate for carboxypeptidase A.[5]

thesized and was investigated as both a substrate and inhibitor for carboxypeptidase A. No measurable enzymic hydrolysis of this D ester could be observed, although it was found to competitively inhibit the enzymic hydrolysis of **2** (R = $C_6H_5CH_2$) with $K_i = 6.3 \times 10^{-3}$ M at pH 7.5, 25 °C, ionic strength 0.2. Thus for **2** (R = C_6H_5) the K_i value for the D isomer is approximately four times larger than K_m for the corresponding L isomer (Table II) derived from the racemic ester as substrate. In general, the use of a racemic ester as substrate, for which only the L isomer is enzymically hydrolyzed while the D isomer acts as a competitive inhibitor, leads to a Michaelis equation having the parameters, $k_{cat}^{app} = k_{cat}/(1 + K_m/K_i)$ and $K_m^{app} = K_m/(1 + K_m/K_i)$, where k_{cat} and K_m are the true parameters for the L isomer alone and K_i is the inhibition constant for the D isomer. In general, provided that K_m is smaller than K_i, $k_{cat}^{app} \simeq k_{cat}$ and $K_m^{app} \simeq K_m$. Also the ratio k_{cat}/K_m is independent of the presence or absence of the D isomer, and K_m^{app} deduced from the racemate must always be equal to or smaller than the true K_m for the L isomer. The above data for the ester **2** (R = C_6H_5) and **2** (R = $C_6H_5CH_2$) and the following discussion all suggest that k_{cat}^{app} and K_m^{app} are close to the true k_{cat} and K_m values for all esters in this study.

p-Nitrobenzoyl-DL-*p*-chloromandelic acid (**2**, R = *p*-ClC_6H_4) was not hydrolyzed at a significant rate even at enzyme concentrations as large as 2.0×10^{-5} M. If this ester is hydrolyzed at all by the enzyme, it is certainly a poorer substrate than any of the other *p*-nitrobenzoate esters listed in Table II. However, this ester did competitively inhibit the hydrolysis of the L isomer of **2** (R = $C_6H_5CH_2$) with $K_i = 1.9 \times 10^{-3}$ M at pH 7.5, 25 °C, ionic strength 0.2.

Studies of the enzymic hydrolysis of **2** (R = $CH_3(CH_2)_5$) were not attempted since solutions of the anion of this ester at concentrations of 0.005 M were quite soapy and so indicated significant micelle formation. It was felt that the presence of this phenomenon would not allow any confidence in the meaning of any kinetic parameters obtained for the enzymic hydrolysis of this ester.

The reversible inhibition of the hydrolysis of **2** (R = $C_6H_5CH_2$) was studied at pH 7.5, 25 °C, and ionic strength 0.2 for a series of carboxylate anions that are known to be reversible inhibitors of the enzymic hydrolysis of *O*-hippuryl-L-3-phenyllactic acid (**3**, R = $C_6H_5CH_2$), a specific ester substrate. All inhibitors examined displayed competitive inhibition kinetics of the enzymic hydrolysis of this *p*-nitrobenzoate ester substrate. Derived inhibition constants, K_i, are collected in Table III. This table also compares the mode of inhibition for these same inhibitors in the hydrolysis of **3** (R = $C_6H_5CH_2$) and peptide substrates.

Discussion

From the data in Table II, it is clear that in the hydrolysis of *p*-nitrobenzoate esters (**2**) by bovine carboxypeptidase A, both k_{cat} and K_m display a pronounced dependence on the nature of the R side chain in the alcohol moiety of **2**. For the homologous series R = H, CH_3, CH_3CH_2, $CH_3(CH_2)_2$, k_{cat} monotonically increases while K_m decreases. Introduction of a further methylene group as in R = $CH_3(CH_2)_3$ then produces a significant decrease in k_{cat} and only a slight further decrease in K_m. A similar pattern is apparent when one compares R = C_6H_5, $C_6H_5CH_2$, and $C_6H_5(CH_2)_2$. Thus introduction of the first methylene unit in this latter series produces an increase in k_{cat} and a decrease in K_m as seen in the above aliphatic series. However, on going from R = $C_6H_5CH_2$ to R = $C_6H_5(CH_2)_2$ there is a marked reversal of these trends, with k_{cat} decreasing and K_m increasing by large amounts. Based on the specificity constant, k_{cat}/K_m, the phenyllactic acid derivative (R = $C_6H_5CH_2$) is clearly the best *p*-nitrobenzoate ester substrate for carboxypeptidase A. The corresponding hippurate ester (**3**, R = $C_6H_5CH_2$) is also the best hippurate ester substrate known for this enzyme.[20]

Taken as a whole, the data in Table II indicate a marked preference in both k_{cat} and K_m for substrates with hydrophobic R side chains in the alcohol unit of **2**, provided that the R group does not become too large (R = $C_6H_5(CH_2)_2$). Such a result suggests that in the vicinity of the enzymic catalytic site at which these esters are hydrolyzed there is a hydrophobic region of limited size which accommodates this R group. While R is small enough to fit into this hydrophobic region in its energetically most favorable conformation, increases in the size of R lead to stronger hydrophobic interactions between enzyme and substrate. This is reflected in a decrease in K_m and also in an increased k_{cat} which may be interpreted in terms of a more rigid binding of the ester carboxylate group in a more favorable orientation for optimal interaction with the catalytically important functional group(s) of the enzyme. The particularly small k_{cat} for *O*-*p*-nitrobenzoylglycolic acid (**2**, R = H) seems to stress the importance of hydrophobic binding of R in assisting the optimal orientation of the ester functional group for maximum catalytic efficiency. Little or no penetration of the hydrophobic region would be expected for R = H, and the very low k_{cat} for this ester is a direct reflection of the lack of hydrophobic bonding to steer the ester functional group into a catalytically efficient site.

When R becomes too large to fit in its energetically most favorable conformation into this hydrophobic region, binding can only occur by utilizing a higher energy conformation of R. Consequently, favorable hydrophobic interactions between enzyme and substrate are offset by energetically unfavorable nonbonded interactions within a high-energy conformation of the R group. These offsetting effects lead to a net decrease in binding energy compared with a lower homologue which binds in its lowest energy conformation and, consequently, to an increase in K_m. The inability of a large R group to fit optimally into the hydrophobic region is also reflected in a decrease in k_{cat} which presumably results from a displacement of the ester functional group into a less than optimal orientation relative to the enzymic catalytic functional group(s).

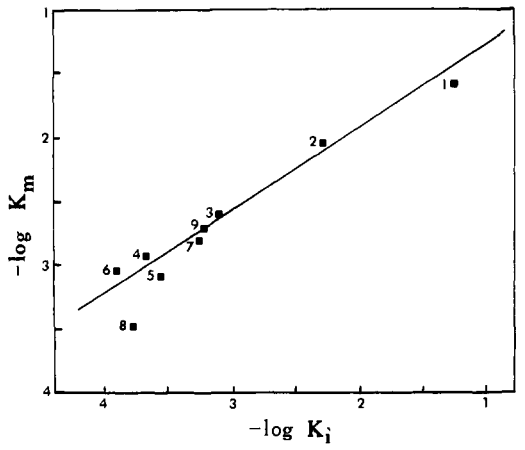

Figure 2. Relationship between log K_m (**2**) and log K_i (**4**). Data for **4** from ref 24. Points are identified by the following R groups: 1-H, 2-CH_3, 3-C_2H_5, 4-$CH_3(CH_2)_2$, 5-$CH_3(CH_2)_3$, 6-$(CH_3)_2CH$, 7-C_6H_5, 8-$C_6H_5CH_2$, 9-$C_6H_5(CH_2)_2$.

The importance of hydrophobic interactions in the binding of small molecules to this enzyme has also been observed for hippuric acid esters (**3**)[20] and for the monocarboxylate anions (**4**)[24] and dicarboxylate anions (**5**)[25] which are competitive

$RCH_2CO_2^-$ **4** $\qquad$ $RCH(CO_2^-)_2$ **5**

inhibitors for the hydrolysis of **3.** For each of **3, 4,** and **5,** the strongest binding, based on K_m for **3** and K_i for **4** and **5,** is observed for $n = 2$ and 3 in the homologous series R = $CH_3(CH_2)_n$; K_i for **4** also passes through a minimum at R = $C_6H_5CH_2$ analogous to K_m for the *p*-nitrobenzoate esters **2.** In fact, there is a linear correlation between log K_m for **2** and log K_i for **4** (Figure 2). For eight R groups, only R = $C_6H_5CH_2$ deviates significantly from the correlation line:

$$\log K_m^2 = 0.61 \log K_i^4 - 0.77 \qquad (r = 0.978)$$

K_m for **2** (R = $C_6H_5CH_2$) is 2.5 times smaller than predicted by this correlation line.

The slope of 0.61 for this correlation line indicates that hydrophobic interactions are less important overall in binding *p*-nitrobenzoate esters than for binding carboxylate ion inhibitors. We have previously noted[24] that when log K_i (**4**) are plotted as a function of π, the Hansch hydrophobicity parameter[30,31] for R, there is an unusually steep linear dependence of log K_i on π. This dependence is approximately $\Delta(\Delta G)$ = 1.4 kcal/mol/CH_2 unit and is too large to represent simply the transfer of a CH_2 group from water to a hydrocarbon environment, and hence may also include an inhibitor dependent conformational change in the EI complex upon inhibitor binding. The slope of the correlation line in Figure 2 suggests that for binding the *p*-nitrobenzoate esters (**2**), each additional CH_2 unit in R is responsible for an increment $\Delta(\Delta G)$ = 0.61 × 1.4 = 0.85 kcal/mol in binding energy. Such a value is close to the usual estimates of the free energy of transfer of a methylene group from water to a hydrocarbon environment.[32,33]

This $\Delta(\Delta G)$ = 0.85 kcal/mol for binding a CH_2 unit of the alcohol side chain of the nonspecific ester substrates (**2**) also contrasts with $\Delta(\Delta G) \simeq$ 1.5 kcal/mol for binding a CH_2 unit of the alcohol moiety of the specific hippurate ester substrates (**3**).[20] If, as we have suggested, such a large $\Delta(\Delta G)$ is related to a conformational change upon binding, then the much larger $\Delta(\Delta G)$ for hippurate esters than for *p*-nitrobenzoate esters indicates that induced fit between the enzyme and substrate is much more important with specific ester substrates than with nonspecific ester substrates. We have previously presented

Table IV. Comparison of *p*-Nitrobenzoate Esters (**2**) and Hippurate Esters (**3**) as Substrates for Carboxypeptidase A.[a]

R	k_{cat}^2/k_{cat}^3	K_m^2/K_m^3	$(k_{cat}/K_m)^2/(k_{cat}/K_m)^3$
H	*b*	*b*	1.5×10^{-3}
CH_3	*b*	*b*	1.9×10^{-3}
C_2H_5	0.017	8.3	2.1×10^{-3}
$CH_3(CH_2)_2$	0.063	32	1.9×10^{-3}
$CH_3(CH_2)_3$	0.024	9.2	2.6×10^{-3}
$(CH_3)_2CH$	0.014	3.8	3.8×10^{-3}
$(CH_3)_2CHCH_2$	0.027	53	5.2×10^{-3}
C_6H_5	0.0045	1.25	3.6×10^{-3}
$C_6H_5CH_2$	0.0056	1.8	3.1×10^{-3}

[a] At pH 7.5, 25 °C, ionic strength 0.5. Data for hippurate esters from ref 20, 34. [b] Individual k_{cat} and K_m values are not experimentally accessible for this hippurate ester.[20,34]

kinetic data that is simply interpretable in terms of an enzymic conformational change upon interaction of the amide unit of the hippuryl moiety of hippurate ester substrates with this enzyme.[18,22] Thus the induced fit of carboxypeptidase A to specific esters seems to be the result of the combined interactions of the acyl moiety and the hydrophobic side chain of the alcohol unit of the substrate with the enzyme. The faster rates of enzymic hydrolysis of specific esters than of nonspecific esters (e.g., k_{cat} for **3** (R = $C_6H_5CH_2$) is approximately 200-fold larger[20] than k_{cat} for **2** (R = $C_6H_5CH_2$)) may reflect the importance of induced-fit conformational effects in securing optimal orientation of the catalytic enzymic functional group(s) toward the hydrolyzed bond in the substrate.

The close correlation observed in Figure 2 between the dissociation constant K_i and K_m strongly suggests that K_m is the true dissociation constant, K_s, of the ES complex, rather than being a more complex combination of rate constants. The only alternative simple interpretation of such a linear correlation would be that K_m is proportional to K_s rather than equal to K_s. Such a situation would require that the ratio k_2/k_1 for

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

should be independent of the nature of the R side chain in **2.** This is possible but unlikely in the light of the pronounced dependence of k_{cat} on R. This correlation also rules out the possibility that decomposition of an acyl-enzyme intermediate is entirely rate determining in these reactions. The dependence of k_{cat} on R and the apparent requirement for $K_m = K_s$ demand that, *if* an acyl-enzyme intermediate is involved in these reactions, then the rate of acylation must be slower than the rate of deacylation.[37]

A comparison of the dependence of the kinetic parameters k_{cat}, K_m, and k_{cat}/K_m on R for *p*-nitrobenzoate esters (**2**) and hippurate esters (**3**) is made in Table IV. Whereas k_{cat}^2/k_{cat}^3 varies over 14-fold and K_m^2/K_m^3 varies 42-fold, the specificity constant ratio $(k_{cat}/K_m)^2/(k_{cat}/K_m)^3$ varies only 3.5-fold for individual variations in k_{cat}/K_m of 10^4-fold for both *p*-nitrobenzoate and hippurate esters. Comparisons of individual k_{cat} and K_m values are always uncertain due to the possibility that some nonproductive binding may be represented by these individual parameters. This does not seem to be important for the *p*-nitrobenzoate esters in the light of the excellent correlation in Figure 2; however, a similar correlation attempted for hippurate esters is much more scattered and the possibilities of nonproductive binding influencing k_{cat} and K_m for these esters seem to be considerable. It has been definitely established that there are at least two binding sites for hippurate esters on carboxypeptidase A.[20,34]

The specificity constant ratio is independent of the effects of nonproductive binding, and the constancy of this ratio for **2** and **3** over the series of R groups in Table IV suggests that at least some important features of substrate binding and/or hydrolysis must be common to these two classes of esters. The common variable in this comparison is the R group, and the importance of the interaction of R with the enzyme in substrate binding is established above for *p*-nitrobenzoate esters and has been established previously for hippurate esters.[20] The constancy of the specificity constant ratio when R is varied in these two classes of esters then strongly suggests that the same enzymic hydrophobic region is involved in an interaction with R in both **2** and **3** and also the carboxylate ion inhibitors **4** and **5** which are competitive inhibitors for both classes of substrate (Table III). This conclusion is further strengthened by the similar effects of a chlorine substituent on the enzymic hydrolysis of **2** (R = *p*-ClC_6H_4) and **3** (R = *p*-ClC_6H_4). Thus the lack of observable enzymic hydrolysis of **2** (R = *p*-ClC_6H_4) corresponds to the 900-fold smaller k_{cat}/K_m ratio observed for **3** (R = *p*-ClC_6H_4) relative to **3** (R = C_6H_5).

It is clear, however, that although the R side chains of the alcohol units of both specific and nonspecific ester substrates bind in the same region of the active site, there must be significant differences in the other interactions of these two classes of substrates with the enzyme. Thus, *p*-nitrobenzoate anion and *N*-chloroacetyl-L-phenylalanine are noncompetitive inhibitors for **3** (R = $C_6H_5CH_2$) (Table III) and so bind at a site which does not overlap with the productive binding site of this specific ester substrate. However, these same two inhibitors display competitive inhibition for **2** (R = $C_6H_5CH_2$) and so must either compete directly for at least part of the binding site for this nonspecific substrate, or alternatively bind at quite a different site and block subsequent substrate binding either by physically preventing passage of the substrate to its productive binding site or alternatively by a conformational change in the EI complex which blocks or destroys the binding site for this substrate.

If one assumes that in all cases competitive inhibition represents competition by substrate and inhibitor for a common enzymic binding site, then Figure 3 gives a minimal schematic representation of the binding of substrates and inhibitors that is consistent with the data in Table III. The minimal requirement is for two sites, A and B, on the enzyme that are able to interact with carboxylate anions. A and B are presumably positively charged. While a common hydrophobic binding site is required for the R groups of specific ester and nonspecific ester substrates, the terminal carboxylate anions of these two classes of substrates are assumed to bind to A and B, respectively (Figures 3a and 3b). Simple carboxylate ions bound as in Figure 3c can then competitively inhibit both classes of ester substrate. Peptide substrates are indicated as binding via interaction of their terminal carboxylate ions with site B (Figure 3d) to satisfy the requirement that *N*-chloroacetyl-L-phenylalanine, which appears to be a typical peptide substrate,[5] is a competitive inhibitor for nonspecific esters, although a noncompetitive inhibitor for specific esters. This latter requirement also indicates that the R side chain of the C-terminal amino acid of peptide substrates must interact with a different region of the enzyme than the R side chain of the alcohol unit of ester substrates. Interaction of the *p*-nitrobenzoate anion with B (Figure 3e) also allows this anion to competitively inhibit nonspecific esters while noncompetitively inhibiting specific ester substrates. A further test of Figure 3 lies in the prediction from Figures 3d and 3e that *p*-nitrobenzoate anion should be a competitive inhibitor for peptide substrates. Competitive inhibition of the hydrolysis of both classes of ester substrates and also peptide substrates by the dianions of dicarboxylic acids (Table III) can be accommodated via interaction of such species with both A and B as in Figure 3f.

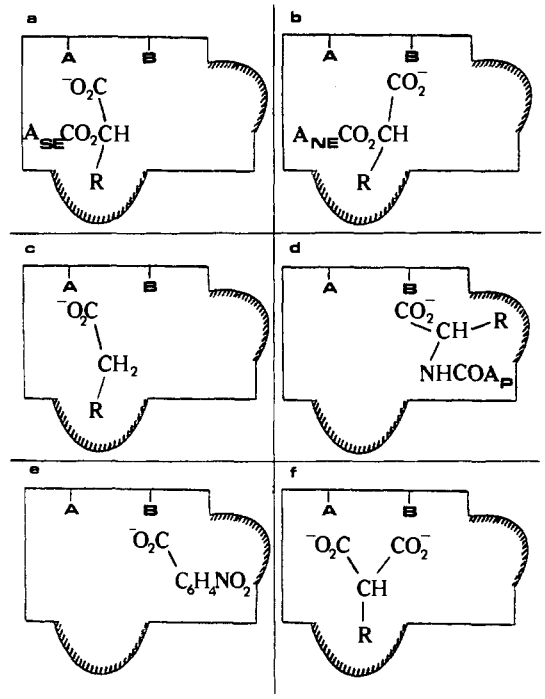

Figure 3. Schematic diagram of the binding of substrates and reversible inhibitors to carboxypeptidase A: (a) specific ester substrates (acyl group represented by A_{SE}); (b) nonspecific ester substrates (acyl group represented by A_{NE}); (c) simple carboxylate ions which are competitive inhibitors of esterase activity and noncompetitive inhibitors of peptidase activity; (d) peptide substrates (acyl group represented by A_P); (e) *p*-nitrobenzoate ion which is a competitive inhibitor for nonspecific esters but a noncompetitive inhibitor for specific esters; (f) malonate dianions which are competitive inhibitors of specific and nonspecific esterase and peptidase activities.

The schematic representation of Figure 3 is consistent with a large body of evidence that is most simply interpreted in terms of distinct binding sites for specific ester and peptide substrates.[15-18] On the basis of a large amount of experimental evidence collected by Vallee, Riordan, Auld, and co-workers, and on consideration of the structure of the enzyme determined by x-ray crystallography,[35,36] it is tempting to conclude that A represents the zinc ion and B represents the guanidinium group of Arg-145. However, an assignment of A and B in this way would seem on the basis of the x-ray crystal structure of the enzyme to place the binding of specific esters and peptides too close together for them to be truly bound in a noninteracting fashion. In particular, the requirement for two different hydrophobic regions is not easily reconciled with this assignment. It should be noted that the guanidinium groups of Arg-71 and Arg-124 are also located in the active site in the vicinity of the zinc ion,[35,36] and we feel that at the present time it is not possible to rule out these guanidinium groups as being possible candidates for A and/or B in Figure 3. It is especially intriguing that Tyr-198 and Phe-279 are located in the vicinity of Arg-71, and this region could provide a suitable hydrophobic environment to accept the R side chain of a substrate bound via its terminal carboxylate ion in an electrostatic interaction to Arg-71. We tentatively suggest that the vicinity of Tyr-198 and Phe-279 and the hydrophobic pocket that has been shown by x-ray crystallography to be present in the vicinity of the zinc ion should be considered as the most likely candidates for the two distinct hydrophobic regions represented in Figure 3.

References and Notes

(1) B. L. Vallee, J. F. Riordan, and J. E. Coleman, *Proc. Natl. Acad. Sci. U.S.A.*, **49**, 109 (1963).
(2) J. F. Riordan and B. L. Vallee, *Biochemistry*, **2**, 1460 (1963).
(3) F. W. Carson and E. T. Kaiser, *J. Am. Chem. Soc.*, **88**, 1212 (1966).

(4) P. L. Hall, B. L. Kaiser, and E. T. Kaiser, *J. Am. Chem. Soc.*, **91**, 485 (1969).
(5) D. S. Auld and B. L. Vallee, *Biochemistry*, **9**, 4352 (1970).
(6) D. S. Auld and B. L. Vallee, *Biochemistry*, **10**, 2892 (1971).
(7) D. S. Auld and B. Holmquist, *Fed. Proc., Fed. Am. Soc. Exp. Biol.*, **31**, 435 Abs (1972).
(8) J. W. Bunting, J. Murphy, C. D. Myers, and G. G. Cross, *Can. J. Chem.*, **52**, 2648 (1974).
(9) E. P. Kang, C. B. Storm, and F. W. Carson, *J. Am. Chem. Soc.*, **97**, 6723 (1975).
(10) J. Suh and E. T. Kaiser, *J. Am. Chem. Soc.*, **98**, 1940 (1976).
(11) J. W. Bunting and S. S.-T. Chu, *Biochemistry*, **15**, 3237 (1976).
(12) E. T. Kaiser, T.-W. Chan, and J. Suh in "Protein-Metal Interactions", M. Friedman, Ed., Plenum Press, New York, N.Y., 1974, pp 49–80.
(13) For a full discussion of this problem see ref 8. The most recent reasonable assignments of pK_a values are given in ref 10.
(14) M. L. Bender and F. J. Kezdy, *Annu. Rev. Biochem.*, **34**, 49 (1965).
(15) J. F. Riordan, *Biochemistry*, **12**, 3915 (1973).
(16) D. S. Auld and B. Holmquist, *Biochemistry*, **13**, 4355 (1974).
(17) L. G. Lange, D. S. Auld, and J. F. Riordan, *Biochemistry*, **13**, 1983 (1974).
(18) J. W. Bunting and C. D. Myers, *Can. J. Chem.*, **53**, 1993 (1975).
(19) Glycolic, L-lactic, D- and DL-mandelic, and L-phenyllactic acids were obtained commercially. DL-3-Phenyllactic and DL-2-hydroxy-4-phenylbutanoic acids were prepared by acidic hydrolysis of the cyanohydrins of phenylacetaldehyde and 3-phenylpropionaldehyde, respectively. Other 2-hydroxy acids were prepared from the commercially available 2-bromo acids.[20]
(20) J. Murphy and J. W. Bunting, *Can. J. Chem.*, **53**, 283 (1975).
(21) Our previous report[8] that the sodium salt of **2** (R = C_6H_5) is insoluble in water at these concentrations is in error. The acetone procedure described herein was not used previously. Our earlier work was complicated by the very low aqueous solubility of the free acid of this ester.
(22) J. W. Bunting and J. Murphy, *Can. J. Chem.*, **52**, 2640 (1974).
(23) G. N. Wilkinson, *Biochem. J.*, **80**, 324 (1961).
(24) J. W. Bunting and C. D. Myers, *Can. J. Chem.*, **51**, 2639 (1973).
(25) J. W. Bunting and C. D. Myers, *Can. J. Chem.*, **52**, 2053 (1974).
(26) J. W. Bunting and C. D. Myers, *Can. J. Chem.*, **53**, 1984 (1975).
(27) S. S.-T. Chu, M.Sc. Thesis, University of Toronto, 1974.
(28) D. S. Auld and B. L. Vallee, *Biochemistry*, **9**, 602 (1970).
(29) L. D. Byers and R. Wolfenden, *Biochemistry*, **12**, 2070 (1973).
(30) C. Hansch, *Acc. Chem. Res.*, **2**, 232 (1969).
(31) A. Leo, C. Hansch, and D. Elkins, *Chem. Rev.*, **71**, 525 (1971).
(32) C. Tanford, *J. Am. Chem. Soc.*, **84**, 4240 (1962).
(33) P. Mukerjee, *Adv. Colloid Interface Sci.*, 1, 241 (1967).
(34) J. Murphy and J. W. Bunting, *Can. J. Chem.*, **52**, 3829 (1974).
(35) W. N. Lipscomb, G. N. Reeke, Jr., J. A. Hartsuck, F. A. Quiocho, P. H. Bethge, M. L. Ludwig, T. A. Steitz, H. Muirhead, and J. C. Coppola, *Brookhaven Symp. Biol.*, **21**, 24 (1968).
(36) W. N. Lipscomb, G. N. Reeke, Jr., J. A. Hartsuck, F. A. Quiocho, and P. H. Bethge, *Philos. Trans. R. Soc. London, Ser. B*, **257**, 177 (1970).
(37) The current status of the acyl–enzyme intermediate hypothesis for carboxypeptidase A is summarized by Suh and Kaiser[10] and Breslow and Wernick.[38]
(38) R. Breslow and D. Wernick, *J. Am. Chem. Soc.*, **98**, 259 (1976).

Intramolecular Catalysis of Acylation and Deacylation in Peptides Containing Cysteine and Histidine

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Abstract: A series of peptides having various arrangements of cysteine and histidine was synthesized to determine the potential of thiol–imidazole intramolecular systems for catalysis of hydrolytic reactions. With substituted phenyl acetates as substrates, no cooperative effect of histidine was observed for the acylation of the cysteine residue. In the deacylation of the S-acylated peptides, again the intramolecular histidine had little or no effect. However, in the presence of the thiol specific reagents, 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent) and *N*-ethylmaleimide, the rates of appearance of free SH from S-acylated peptides were much greater than the observed deacylation rates in the absence of these reagents. For the peptide N-Ac-Gly-Arg-Phe-Cys(Ac)-Phe-His-Gly-COOH, the rate constant for the appearance of free SH in the presence of Ellman's reagent was 3.1×10^{-2} min^{-1} under conditions for which the observed deacylation rate was 3.2×10^{-4} min^{-1} (pH 8.1, 25 °C). This trapping effect was not observed for cysteine peptides lacking histidine or for mixtures of *N*,*S*-diacetylcysteine and imidazole free in solution. These observations indicate the existence of a rapid, reversible intramolecular transfer of the acetyl group between the cysteine and histidine residues which greatly favors cysteine. A mechanism inhibiting back-attack by cysteine of the acyl histidine would allow efficient deacylation to occur through intramolecular nucleophilic catalysis by the imidazole group. The cysteine proteases may have evolved such a mechanism.

There exists in nature a homologous series of proteolytic enzymes having cysteine and histidine at their active sites. In these enzymes, such as papain, the thiol group of cysteine serves as the primary nucleophile, cleaving the acyl linkage of the substrate with the resultant formation of an acyl-thiol intermediate. The imidazole moiety of histidine is thought to assist in this process as well as in the deacylation of the acyl-enzyme required to regenerate the catalyst.[1]

A number of model thiol–imidazole intramolecular systems have been studied previously in efforts to demonstrate the catalytic ability of this combination for hydrolytic reactions. With cysteine–histidine peptides,[2-6] little or no assistance by imidazole has been found for the nucleophilic attack of the SH group on *p*-nitrophenyl acetate. With the same substrate, only a sixfold increase in rate was observed[6] for the reaction with 4-(2-mercaptoethyl)imidazole over that with glutathione.

For the deacylation step the largest acceleration reported was 30-fold, found for S-Ac-*cyclo*-(Cys-His) compared with ethyl thiolacetate.[6] However, as shown below, when comparisons are made to more appropriate models lacking imidazole, little if any of this effect can be attributed to the imidazole group.

Thus, there has yet to be demonstrated any significant cooperative effect in the catalysis of hydrolytic processes by thiol–imidazole intramolecular systems as is thought to occur in the cysteine proteases. Since in certain of these model systems the imidazole and SH groups are in extremely close proximity, interactions with other functional groups must be required for the imidazole moiety to exert its catalytic effects. With this in mind we studied a series of cysteine–histidine peptides having other functional groups, looking not only for overt catalytic efficacy, but also for potential catalytic effects with thiol-specific trapping reagents. Our results show the nature and magnitude of the intramolecular effects that do