The Hydrolysis of Benzoate Esters by Carboxypeptidase A and the p*H*-Rate Profile for the Hydrolysis of *O*-Hippuryl-L-3-phenyllactic Acid

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A series of *para*-substituted *O*-benzoyl-2-hydroxybutanoic acids (but not the unsubstituted ester) are hydrolyzed by bovine carboxypeptidase A (p*H* 7.5, ionic strength 0.5, 25°). For the CH_3O, CH_3, Cl, CN, and NO_2 substituents, there exist linear correlations of k_{cat} and K_m with the Hammett σ constants for these substituents ($\log k_{cat} = 1.17\sigma + 1.17$; $\log K_m = -0.53\sigma - 2.15$), although the *tert*-butyl group shows significant deviations from both correlation lines. The above unsubstituted ester is a reversible inhibitor of the enzymic hydrolysis of *O*-hippuryl-L-3-phenyllactic acid and so the lack of observable hydrolysis of this ester is attributable to nonproductive binding. The p*H*–rate profiles for k_{cat}/K_m and k_{cat} have been determined for the enzymic hydrolysis of *O*-(*p*-nitrobenzoyl)mandelic acid ($pK_{EH_2} = 6.95$, $pK_{EH} = 7.9$ and $pK_{EH_2S} = 7.5$, $pK_{EHS} = 8.3$) and *O*-hippuryl-L-3-phenyllactic acid ($pK_{EH_2} = 5.8$, $pK_{EH} = 9.3$). For the latter ester k_{cat} is p*H* independent in the range p*H* 5–10. The mechanism of ester hydrolysis catalyzed by carboxypeptidase A is discussed in the light of the above observations and the known crystal structure of the enzyme. A definition of specific and nonspecific substrates for this enzyme based on the observed p*H* profiles for k_{cat}/K_m is proposed.

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Des séries d'acides *O*-benzoyl hydroxy-2 butanoïques substitués en *para* (mais pas les esters non substitués) sont hydrolysés par la carboxypeptidase A du bovin (p*H* 7.5, 25°, force ionique de 0.5). Il existe des corrélations linéaires entre k_{cat}, K_m et les constances σ de Hammett des substituants CH_3O, CH_3, Cl, CN et NO_2 ($\log k_{cat} = 1.17\sigma + 1.17$; $\log K_m = -0.53\sigma - 2.15$). Toutefois le groupe *tert*-butyle montre des déviations significatives de ces deux droites de corrélations. L'ester non substitué ci-dessus est un inhibiteur réversible de l'hydrolyse enzymatique de l'acide *O*-hippuryl phényl-3 L-lactique, et ainsi on n'observe pas d'hydrolyse de cet ester à cause d'une liaison non-réactive. On détermine la courbe de p*H* pour k_{cat}/K_m et k_{cat} lors de l'hydrolyse enzymatique de l'acide *O*-(*p*-nitrobenzoyl) mandélique ($pK_{EH_2} = 6.95$, $pK_{EH} = 7.9$ et $pK_{EH_2S} = 7.5$, $pK_{EHS} = 8.3$) et de l'acide *O*-hippuryl phényl-3 L-lactique ($pK_{EH_2} = 5.8$, $pK_{EH} = 9.3$). Pour ce dernier ester, k_{cat} reste constant pour une variation du p*H* de 5–10. A la lumière de ces observations et de la structure connue du cristal de l'enzyme, on discute du mécanisme de l'hydrolyse des esters catalysée par la carboxypeptidase A. On propose une définition des substrats spécifiques et non-specifiques pour cette enzyme, basée sur l'observation des courbes de p*H* pour k_{cat}/K_m. [Traduit par le journal]

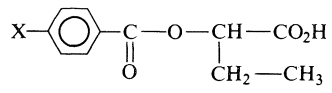
During the past few years, evidence has accumulated that the γ-carboxyl group of glutamic acid residue 270 plays a role in the mechanism of hydrolyses catalyzed by bovine carboxypeptidase A. Lipscomb and co-workers (1–4), based on their X-ray crystallographic investigation of the structure of this enzyme, originally suggested that this carboxylate ion may be a general-base or nucleophilic catalyst for the hydrolysis of ester and amide substrates. Following upon this suggestion, a number of reagents were observed to irreversibly inhibit both the peptidase and esterase activities of this enzyme by reaction with the glu-270 γ-carboxyl group. Thus, the irreversible inhibition of the enzyme by both *N*-ethyl-5-phenylisoxazolium-3′-sulfonate (Woodward's reagent K) and *N*-bromoacetyl-*N*-methyl-L-phenylalanine has been unambiguously shown (5–8) to be due to modification of glu-270, while 1-cyclohexyl-3-(2-*N*-methylmorpholinioethyl)carbodiimide *p*-toluenesulfonate is also believed (9) to cause inhibition by modification of this same side chain.

Experiments designed to distinguish between a general-base and nucleophilic role for the glu-270 γ-carboxylate ion have not to date provided an unambiguous interpretation. A direct nucleophilic attack by the carboxylate

ion on the carbonyl carbon of the hydrolyzable bond of the substrate predicts the formation of a mixed anhydride species as an acyl-enzyme intermediate. Attempts to spectrophotometrically observe such a species (10) or to trap it through transpeptidation or transesterification reactions (11, 12) have been unsuccessful. The lack of positive results in such experiments does not, of course, rule out the existence of an anhydride intermediate, which, if formed, would be expected to be highly reactive towards aqueous solvent. In principle, nucleophilic and general-base mechanistic pathways could also be distinguished by the observation of a relatively large solvent isotope effect in the latter case. Studies in H_2O and D_2O indicate that $k_H/k_D = 1.1–1.2$ for peptide substrates (13–15) and $k_H/k_D \sim 2.0$ for ester substrates (15, 16). This data has been tentatively interpreted as supporting a general-base mechanism for ester hydrolysis; however, the usual complications (17) that obscure the interpretations of isotope effects in enzymic reactions cannot be eliminated and other experimental approaches to this problem are desirable.

In considering this problem, we were attracted by the observation of Hubbard and Kirsch (18) that the Hammett ρ values for nucleophilic attack on substituted benzoate esters show a strong dependence on the charge of the attacking nucleophile (*i.e.* anionic or neutral). Within these two classes of nucleophiles, ρ seems to be relatively independent of the chemical nature of the nucleophile, in both enzymic and nonenzymic systems. Thus, provided a suitable series of benzoate ester substrates can be found, the ρ values for the carboxypeptidase A-catalyzed hydrolysis of such esters may provide an insight into the charge type of the enzymic nucleophile and thus allow a decision on a nucleophilic (anionic nucleophile) or general-base (neutral nucleophile-water molecule) role for glu-270.

In the present work, we have synthesized a series of *para*-substituted *O*-benzoyl-2-hydroxybutanoic acids (**1**) and have shown that, in general, these esters are hydrolyzed by bovine carboxypeptidase A. In addition, we have measured the p*H*–rate profiles for the hydrolysis of *O*-(*p*-nitrobenzoyl)mandelic acid (**2**) and *O*-hippuryl-L-3-phenyllactic acid by this enzyme. Clearly, any mechanistic comparison of the hydrolysis of benzoate and hippurate ester substrates by this enzyme would rely on a similarity in the p*H*–rate profiles for these two



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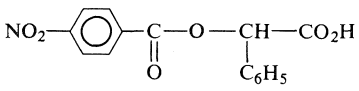


2

classes of substrates. The ester **2** was chosen for a complete p*H* study to allow a more direct comparison with *O*-acetylmandelic acid which has been extensively studied by Carson and Kaiser (41).

Experimental

Substrates

The racemic esters, **1**, were prepared by refluxing the triethylammonium salt of the appropriate *para*-substituted benzoic acid with either *tert*-butyl DL-2-bromobutanoate (X = Cl, CN, NO_2) in nitromethane or benzyl DL-2-bromobutanoate (X = H, CH_3, CH_3O, $(CH_3)_3C$) in acetonitrile followed by removal of the *tert*-butyl (*p*-toluenesulfonic acid in benzene) or benzyl (hydrogenation) protecting groups. The detailed experimental procedures for these preparations closely followed the routes previously described for the synthesis of other ester substrates for this enzyme (19, 20). Melting points, p.m.r. spectra, and elemental analyses of these esters are collected in Table 1.

O-(p-Nitrobenzoyl)-DL-mandelic Acid

A xylene solution (100 ml) of *p*-nitrobenzoyl chloride (9.3 g) was slowly added dropwise to a refluxing solution of mandelic acid (7.6 g) in xylene (30 ml). After refluxing for a further 12 h, the solution was cooled and a small amount of precipitated *p*-nitrobenzoic acid was removed. The solution was concentrated on the rotary evaporator, whereupon the desired ester precipitated. This crude product (m.p. 160–164°) was separated and recrystallized twice from benzene; yield 27%; m.p. 167–169°; p.m.r. (acetone): δ 6.20 (s, 1H), 7.3–7.75 (m, 5H), 8.32 (s, 4H).

Anal. Calcd.: C, 59.9; H, 3.66; N, 4.65. Found: C, 59.5; H, 3.73; N, 4.93.

O-Hippuryl-L-3-phenyllactic acid was a product of Fox Chemical Co., Los Angeles.

Carboxypeptidase A was obtained as a suspension preserved with toluene from Worthington Biochemical Corp. (Code COA). Stock solutions of this enzyme were prepared as previously described (21).

Kinetic Measurements

All hydrolyses were followed on a Radiometer p*H*-Stat in aqueous solution at 25° and p*H* 7.5 unless otherwise indicated. The general experimental technique and estimation of kinetic parameters were as previously described (21). Studies on *O*-hippuryl-L-3-phenyllactic acid and *O*-(*p*-nitrobenzoyl)mandelic acid are at ionic strength 0.2 (NaCl), while the benzoate esters (**1**) were investigated at ionic strength 0.5 (NaCl). It was found

TABLE 1. Characterization of the esters X—$C_6H_4CO_2CH(CH_2CH_3)CO_2H$ (**1**)

X	Melting point (°C)	Proton magnetic resonance (δ in $CDCl_3$)						Analysis (%) Calcd.			Found		
		CH_3[a]	CH_2[b]	CH[c]	Aromatic	OH	Other	C	H	N	C	H	N
H	81	1.12	2.07	5.23	7.43, 8.07[d]	11.57	—	63.5	5.77	—	63.3	5.76	—
CH_3	93	1.10	2.05	5.22	7.22, 7.95[e]	11.10	2.40[f]	64.9	6.31	—	65.5	6.50	—
CH_3O	79	1.08	2.03	5.18	6.88, 8.00[e]	11.00	3.83[f]	60.5	5.88	—	60.3	5.88	—
$(CH_3)_3C$	109–110	1.10	2.05	5.23	7.48, 8.06[e]	9.87	1.35[g]	68.2	7.58	—	68.6	7.70	—
Cl	86–87	1.10	2.03	5.20	7.39, 8.00[e]	10.60	—	54.4	4.57	—	54.4	4.79	—
CN	124–126	1.10	2.07	5.23	7.72, 8.15[e]	11.28	—	61.8	4.75	6.02	61.3	4.66	5.94
NO_2	117–118	1.20	2.17	5.33	8.30[h]	12.62	—	52.2	4.38	5.53	52.1	4.50	5.53

[a]3H,t,J = 7 Hz.
[b]2H,m.
[c]1H,t,J = 6 Hz.
[d]3H and 2H multiplets, respectively.
[e]Both 2H,d,J = 9 Hz.
[f]3H,s.
[g]9H,s.
[h]4H,s.

necessary to work in the presence of 5% dimethyl sulfoxide in order to attain suitable concentrations of **2** since even the sodium salt of this ester proved to be quite insoluble in aqueous solution. Stock solutions of the free acid of **2** were prepared in dimethyl sulfoxide. Suitable aliquots of this solution plus sufficient dimethyl sulfoxide to give a final composition of 5% dimethyl sulfoxide (v/v) in the reaction solution were added to an aqueous solution containing sufficient sodium hydroxide to neutralize the substrate carboxylic acid moiety and sufficient sodium chloride to give a total ionic strength 0.2. Enzyme concentrations for the benzoate ester hydrolyses were in the range 1×10^{-6} to 3×10^{-5} M.

For each of the *para*-substituted benzoate esters only 50 ± 2% of the racemic esters were hydrolyzed at equilibrium. This is consistent with the general observation (22–25) that only the L-enantiomers of substrates of this enzyme are hydrolyzed and all data and calculations are therefore based on the assumption that the D-esters are neither substrates nor competitive inhibitors. In all cases nonenzymic hydrolysis rates were observed to be less than 1% of the enzyme-catalyzed rates.

Results

Each of the esters **1**: X = CH_3, CH_3O, Cl, CN, NO_2, $(CH_3)_3C$ was hydrolyzed by carboxypeptidase A at pH 7.5. Lineweaver–Burk plots for each of these esters are shown in Fig. 1. These plots are clearly linear at low substrate concentrations but positive deviations are observed at high substrate concentrations for X = CH_3O, CH_3, Cl, and $(CH_3)_3C$. This behavior is typical of substrate inhibition and is observed with many ester and peptide substrates of this enzyme (22, 23, 26, 27). In all cases the concentrations at which substrate inhibition becomes important are sufficiently large that reasonably accurate estimates of K_m and k_{cat} can be obtained by fitting the linear portions of these graphs. Values of these two parameters are collected in Table 2.

Surprisingly, no hydrolysis of the unsubstituted ester, **1**: X = H, could be observed under the same conditions as were used for the substituted benzoate esters, or even at enzyme concentrations as great as 10^{-5} M. The ester **1**: X = H, does bind to the enzyme, however, since it reversibly inhibits the enzymic hydrolysis of *O*-hippuryl-L-3-phenyllactic acid (Fig. 2). The inhibition does not appear to take a simple competitive or noncompetitive form, however, and we have been unable to evaluate an inhibition constant for this ester.

From the data in Table 2, it is clear that each of K_m, k_{cat}, and k_{cat}/K_m for the esters, **1**, shows a dependence on the substituent X. Plots of each of these parameters against the Hammett σ substituent constant (28) are indicated in Fig. 3. In each case, there appears to be a linear correlation with σ for five substituents, although the *tert*-butyl group shows a large deviation from each of the correlation lines. Least-squares fitting (ignoring the *tert*-butyl data points) gives the equations:

$$\log k_{cat} = 1.17\sigma + 1.17 \quad (r = 0.985)$$

$$\log K_m = -0.53\sigma - 2.15 \quad (r = 0.943)$$

$$\log (k_{cat}/K_m) = 1.71\sigma + 3.31 \quad (r = 0.998)$$

O-(*p*-Nitrobenzoyl)mandelic acid (**2**) also proved to be a reasonable substrate for bovine carboxypeptidase A. The Lineweaver–Burk plot

TABLE 2. Hydrolysis of *p*-X—$C_6H_4CO_2CH(CH_2CH_3)CO_2H$ by carboxypeptidase A[a]

X	k_{cat} (min^{-1})	K_m (M)	k_{cat}/K_m (M^{-1} min^{-1})
CH_3O	5.4±0.7	$(8.5\pm1.9)\times10^{-3}$	6.3×10^2
$(CH_3)_3C$	68±3	$(2.4\pm0.2)\times10^{-3}$	2.8×10^4
CH_3	10±2	$(8.4\pm2.2)\times10^{-3}$	1.2×10^3
Cl	37±1	$(7.7\pm0.3)\times10^{-3}$	4.9×10^3
CN	79±2	$(2.8\pm0.2)\times10^{-3}$	2.8×10^4
NO_2	111±3	$(2.5\pm0.2)\times10^{-3}$	4.4×10^4
NO_2[b]	54±9	$(1.3\pm0.2)\times10^{-3}$	4.2×10^4

[a]At p*H* 7.5, 25°, ionic strength 0.5.
[b]5% dimethyl sulfoxide – water, other conditions as above.

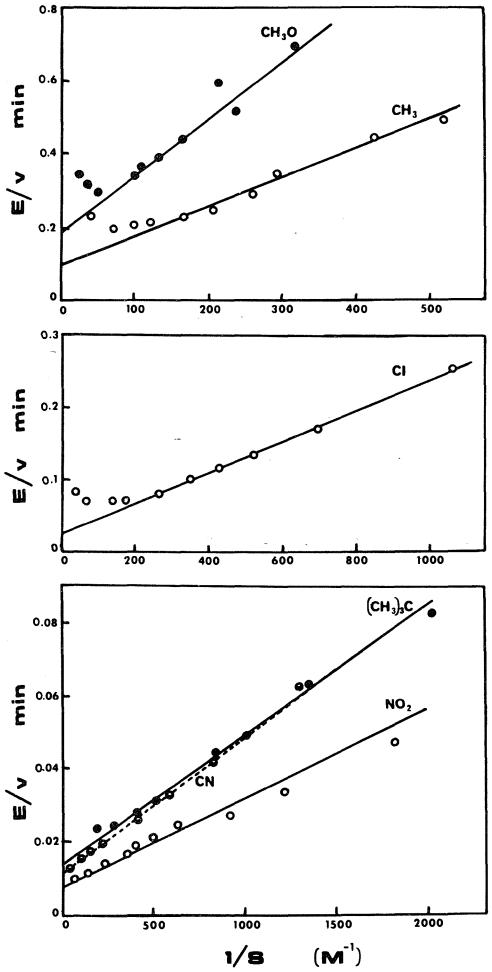


FIG. 1. Hydrolysis of esters **1** by carboxypeptidase A at p*H* 7.5, 25°, ionic strength 0.5; (substituent X is indicated on each line).

for this ester at p*H* 7.5 (k_{cat} = 167 min^{-1}, $K_m = 1.3\times10^{-3}$ *M*) in Fig. 4 also indicates that substrate inhibition is important at high substrate concentrations in this case. A complete

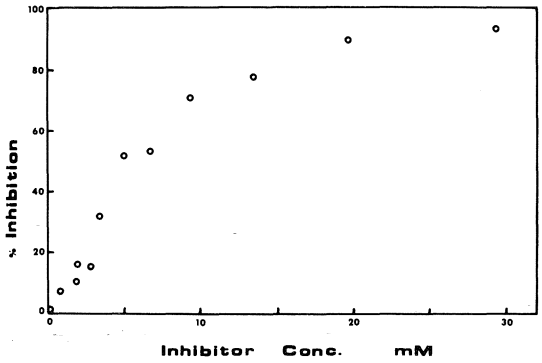


FIG. 2. Inhibition of the hydrolysis of *O*-hippuryl-L-3-phenyllactic acid (5.5×10^{-5} *M*) by **1**: X = H.

p*H*–rate profile for this ester was also determined at 25°. The dependences of k_{cat}/K_m and k_{cat} on p*H* are indicated in Fig. 5. Fitting of the data in Fig. 5 to the equations:

$$[1]\qquad k_{cat}/K_m = \frac{k_2/K_S}{1 + [H^+]/K_{EH_2} + K_{EH}/[H^+]}$$

and

$$[2]\qquad k_{cat} = \frac{k_2}{1 + [H^+]/K_{EH_2S} + K_{EHS}/[H^+]}$$

by least-squares analysis according to

$$\frac{K_m}{k_{cat}}[H^+] = \frac{[H^+]^2}{K_{EH_2}}\frac{K_S}{k_2} + [H^+]\frac{K_S}{k_2} + K_{EH}\frac{K_S}{k_2}$$

and

$$\frac{[H^+]}{k_{cat}} = \frac{[H^+]^2}{K_{EH_2S}}\frac{1}{k_2} + \frac{[H^+]}{k_2} + \frac{K_{EHS}}{k_2}$$

gave the curves indicated in Fig. 5, with pK_{EH_2} = 6.95, pK_{EH} = 7.9, pK_{EH_2S} = 7.5, pK_{EHS} = 8.3, k_2 = 260 min^{-1}, and $K_S = 3.0\times10^{-3}$ *M*. The various ionization constants are defined by Scheme 1, which is the simplest general scheme consistent with the observed p*H* dependences.

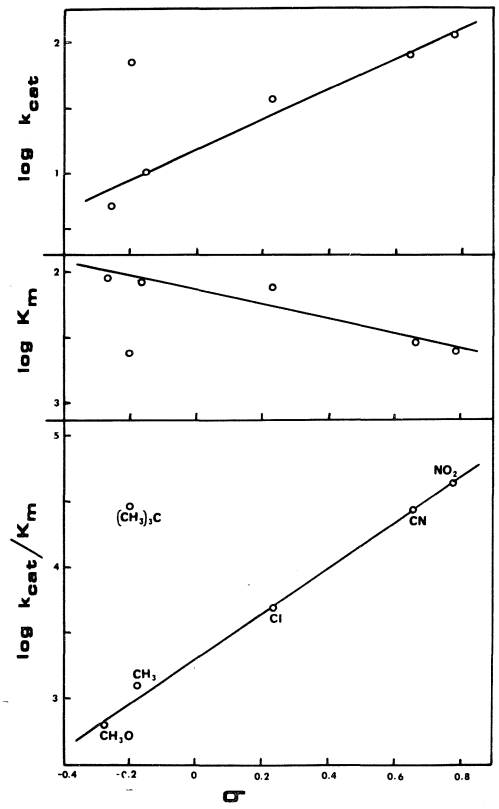


FIG. 3. Dependence of k_{cat}, K_m, and k_{cat}/K_m for the enzymic hydrolysis of **1** on the Hammett σ constant.

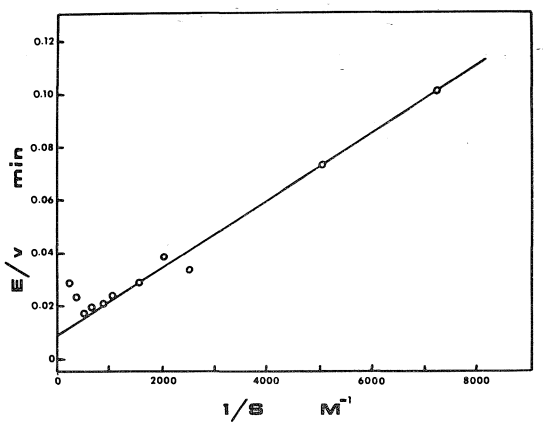


FIG. 4. Hydrolysis of **2** by carboxypeptidase A at p*H* 7.5, 25°, ionic strength 0.2 in 5% dimethyl sulfoxide – water.

The p*H*–rate profile for the carboxypeptidase A-catalyzed hydrolysis of *O*-hippuryl-L-3-phenyllactic acid has also been determined. Typical Lineweaver–Burk plots for the hydrolysis of this ester at a number of p*H* values are indicated in

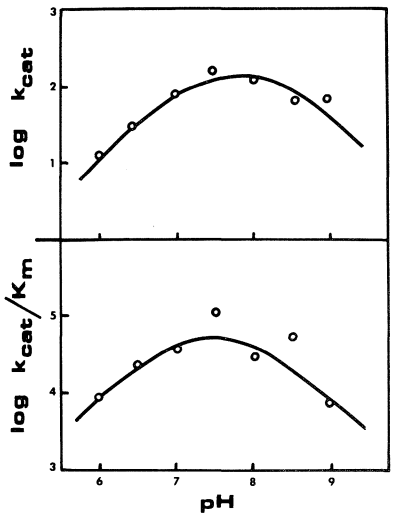
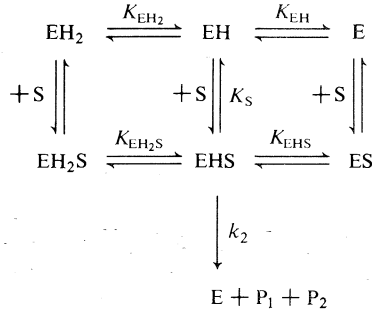


FIG. 5. The p*H* dependence of k_{cat} and k_{cat}/K_m for the hydrolysis of **2** by carboxypeptidase A. Curves are calculated as indicated in the text.



SCHEME 1

Fig. 6. The p*H* profiles for k_{cat} and k_{cat}/K_m for this ester are indicated in Fig. 7. The simplest general scheme applicable to these observed p*H* profiles is outlined in Scheme 2, which is a particular case of Scheme 1 but takes into account the observed p*H* independence of k_{cat} ($= k_2$).[1] Fitting k_{cat}/K_m to eq. 1 by means of the broken lines of slope 1, 0, and −1 in Fig. 7 allows the estimation of $pK_{EH_2} = 5.8$, $pK_{EH} = 9.3$, $k_2 = 3.5 \times 10^4$ min^{-1}, and $K_S = 1.1 \times 10^{-4}$ *M* which have been used to compute the curve in Fig. 7.

Although this ester is by far the most widely studied ester substrate of this enzyme, previous investigators seem to have been dissuaded from

[1]The p*H* independence of k_{cat} could also arise from an alternative, but less likely, modification of Scheme 1, in which EH_2S, EHS, and ES are all catalytically active with identical rate constants, k_2.

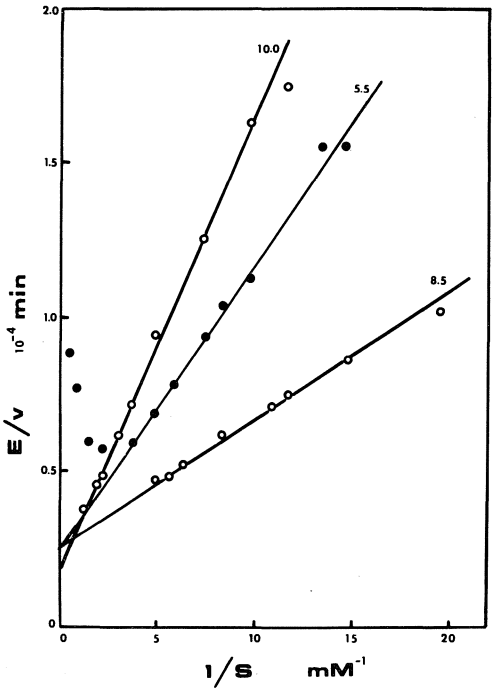
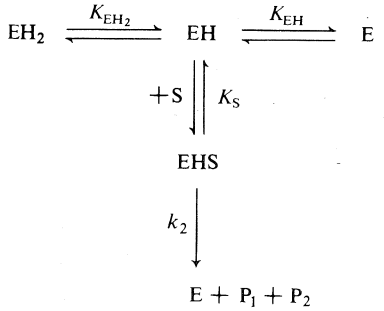


FIG. 6. Hydrolysis of *O*-hippuryl-L-3-phenyllactic acid by carboxypeptidase A at selected p*H* values (25°, ionic strength 0.2).



SCHEME 2

attempting a detailed study of the p*H*–rate profile for this ester by the substrate inhibition effects which intrude (22, 23, 26). As a result, relatively uninformative p*H* investigations at only one substrate concentration have previously been attempted (29). Our data indicate that this phenomenon does not, in fact, produce the anticipated limitations on a complete p*H* investigation. Thus, above the optimal p*H* 7.5, the occurrence of substrate inhibition is displaced to higher substrate concentrations, while at p*H* < 7.5, although pronounced substrate inhibition is still present, it does not seriously interfere in the estimation of the catalytic parameters, although at p*H* 4.55 only k_{cat}/K_m could be accurately obtained.

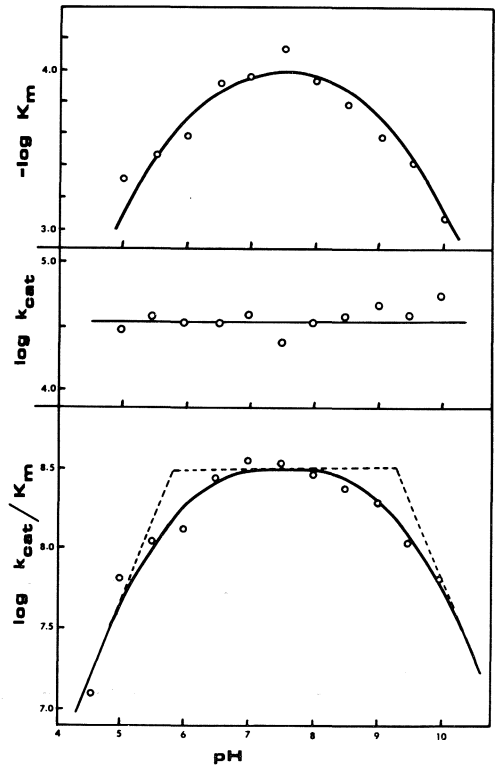


FIG. 7. The p*H* dependence of K_m, k_{cat}, and k_{cat}/K_m for the hydrolysis of *O*-hippuryl-L-3-phenyllactic acid by carboxypeptidase A.

Discussion

The esters **1** and **2** are the first esters of benzoic acid found to be substrates for bovine carboxypeptidase A. All these benzoate esters are relatively poor substrates in terms of both k_{cat} and k_{cat}/K_m compared with this enzyme's most studied ester substrate, *O*-hippuryl-L-3-phenyllactic acid. However, most of these benzoate esters are as good as or better substrates than the extensively studied *O*-acetyl-L-mandelic acid (k_{cat} = 33 min^{-1}, k_{cat}/K_m = 400 M^{-1} min^{-1} (25) at p*H* 7.5, 25°, ionic strength 0.5).

Comparison of the data in Table 1 for **1**: X = NO_2 in water and in 5% dimethyl sulfoxide–water suggests that the presence of this cosolvent tends to decrease both k_{cat} and K_m by a factor of approximately 2, and so has no net effect on k_{cat}/K_m. Thus the ratio k_{cat}/K_m for *O*-(*p*-nitrobenzoyl)mandelic acid should be directly comparable with the specificity constants for other esters in aqueous solution.

Significance of the ρ Parameters

Figure 3 clearly indicates a close correlation of both k_{cat} and K_m with σ for five substituents X in ester **1** which vary in electronic properties from CH_3O to NO_2. The deviation of X = *tert*-butyl from these correlation lines will be considered later. The value of $\rho(k_{cat}) = 1.17$ for the correlation of k_{cat} with σ is somewhat lower than is observed for correlations of nonenzymic base-catalyzed hydrolyses of benzoate esters: $\rho(k_{OH^-}) = 1.93$ for methyl benzoates in 33% dioxane–water (30); $\rho(k_{OH^-}) = 2.01$ for *p*-nitrophenylbenzoates in 33% acetonitrile–water (31). The value of $\rho(k_{cat})$ is, however, much greater than is typical of nonenzymic acid-catalyzed ester hydrolyses: $\rho(k_{H^+}) = 0.11$ for methyl benzoates in 60% acetone–water (32).

Hubbard and Kirsch (18) have compiled ρ values for nucleophilic attack on acyl-substituted *p*-nitrophenylbenzoates by a series of nucleophiles. The ρ values appear to fall into two groups depending only upon whether the nucleophile is anionic or neutral. Thus for neutral nucleophiles ρ = 1.0–1.4, while for anionic nucleophiles ρ = 1.8–2.0, with each class containing both oxygen and nitrogen nucleophiles of various chemical type. These investigators quite reasonably explain this difference in terms of a greater contribution from electron-withdrawing substituents to the stabilization of anionic transition states for nucleophilic attack relative to the stabilization of analogous neutral transition states. If we assume[2] that a similar relationship also holds for attack by anionic and neutral nucleophiles on alkyl benzoates then the above comparison of $\rho(k_{cat})$ and $\rho(k_{OH^-})$ values in this context clearly suggests that this enzymic reaction involves attack on the carbonyl carbon of the ester substrate by a *neutral* nucleophile.

An alternative interpretation of $\rho(k_{cat}) = 1.17$ is evident, however. If attack by an anionic nucleophile was assisted by general-acid catalysis, one might predict that the observed ρ value would be considerably smaller than normally observed for anionic nucleophiles. Such a prediction would result from the base-weakening effect for protonation of the ester carbonyl group that would be caused by electron-withdrawing substituents in the benzoic acid moiety. Since donation of a proton to the ester oxygen atom via general-acid catalysis will be related to the basicity of the accepting atom, we would therefore have a situation in which ρ will be positive for the nucleophilic attack component but negative for the general-acid proton donation component. The net result will be a reduction in $\rho(k_{cat})$ from the value normally observed for anionic nucleophilic attack and the possibility that this value would fortuitously fall in the range associated with the attack of neutral nucleophiles that are unassisted by general-acid catalysts.[3] The observed p*H* dependence of k_{cat} for both *O*-acetyl- and *O*-(*p*-nitrobenzoyl)mandelic acids on the base form of an enzymic acid ($pK_a \sim 7$) and on the acid form of another acidic group ($pK_a \sim 8$) is also suggestive of this interpretation.

A choice between these two possible interpretations for $\rho(k_{cat}) = 1.17$ can ultimately only be made in the context of the catalytic groups that are available at the active site of the enzyme to play general-acid and general-base nucleophilic roles. Although the amino acid side chains that are present in the vicinity of the active site of this enzyme have been clearly indicated by the X-ray crystallographic investigations of Lipscomb and co-workers (1–4), the mode of binding of substrate molecules in this active-site region is still uncertain. Suggestions (3, 4) have been made from model-building studies that are based on the observed binding to the crystalline enzyme of the very poor substrate, glycyl-L-tyrosine. For both ester and peptide substrates, the favoured modes of binding are such that the side-chain carboxylate group of glu-270 is attractively situated for direct nucleophilic attack on the carbonyl carbon atom of the hydrolyzable bond or for general-base catalysis of the nucleophilic attack by a water molecule on this same bond. Furthermore, in these substrate binding modes, the phenolic OH of tyr-248 is suitably located so that it could donate

[2]The fact that $\rho(k_{OH^-})$ for methyl and *p*-nitrophenylbenzoates are not very different (see above) suggests that the ρ value is not strongly dependent on the nature of the alcohol leaving group (*cf.* $\rho(k_{OH^-}) = 2.2$ for the even more highly activated 2,4-dinitrophenylbenzoates (18)), and therefore this assumption appears quite reasonable.

[3]The extreme case of such general-acid catalysis can be considered to be specific-acid catalysis for which ρ values close to zero (33) are most often observed. In this case the electronic effects of a substituent in the phenyl ring upon susceptibility to nucleophilic attack on the one hand, and basicity on the other, are approximately offsetting.

a proton as a general-acid catalyst in the transition state for nucleophilic attack. Thus, functional groups would seem to be available to play each of the catalytic roles in the above two suggested interpretations of $\rho(k_{cat}) = 1.17$, provided that the postulated substrate binding modes are correct.

A metal-coordinated water molecule is an alternative possible candidate for a general-acid catalyst role in the above mechanistic interpretation. This would require the assignment of the $pK_a \sim 8.0$ in the E.S complex to the deprotonation of this water molecule. Although in principle the coordinated water molecule may alternatively be able to play the role of a neutral nucleophile, this is unlikely to be the case, since the coordinated hydroxide ion that is produced upon deprotonation should then be a much better nucleophile, and so an enhanced rate of hydrolysis in basic solutions should result. This is not observed in the present cases (Figs. 5 and 7), although a pronounced increase in k_{cat} in basic solutions is observed in the enzymic hydrolysis of *O*-cinnamoyl-L-3-phenyllactic acid (34).

The results of chemical modification studies of this enzyme indicate that modification of tyr-248 in any way, including acetylation of the OH group, destroys peptidase activity but results in activation of the enzyme for hydrolysis of specific ester substrates (23, 26, 29). Much of this activation of esterase activity is due to the relief of substrate inhibition effects. However, for the hydrolysis of *O*-hippuryl-L-3-phenyllactic acid there is a modest increase (two- to three-fold (23, 26)) in k_{cat} for the acetyl-enzyme compared with the native enzyme, and this clearly indicates that a mechanism involving this phenolic group as a general-acid catalyst is unacceptable for the hydrolysis of this ester. Also, K_m for this substrate is increased 30–40-fold upon acetylation (23, 26), and this latter observation is noteworthy in considering the brief report (15) that acetylation of tyr-248 also abolishes the enzymic hydrolysis of the ester substrate, *O*-acetylmandelic acid. Although this indicates an apparent difference in the esterase activity of the acetyl-enzyme towards these two ester substrates, it should be noted that a 40-fold increase in K_m for the latter ester with the acetyl-enzyme over that observed for the native enzyme ($K_m = 0.08\ M$ (25)) would probably lead to undetectable hydrolysis of this poor substrate due to the low concentrations of the relatively unreactive E.S complex that would be present at the usual substrate concentrations.

Thus, we do not feel that the available evidence upon the effects of acetylation of tyr-248 can be used to support a difference in the binding modes of specific and nonspecific ester substrates.[4] On the other hand, the quite different p*H* profiles for k_{cat}/K_m for specific and nonspecific substrates do seem to indicate that the more basic group, at least, that controls substrate binding is different for these two classes of substrates. This must then reflect differences in the binding of specific and nonspecific substrates, and also possibly different catalytic mechanisms for the hydrolysis of these two classes of esters.

The small negative ρ value for K_m clearly indicates that if K_m is a true reflection of the E.S dissociation constant (*i.e.* $K_m = K_S$), then the ester is not bound by coordination of the carbonyl oxygen atom to the zinc ion as has been proposed for specific substrates (3, 4). Such coordination would be expected to be reduced by the presence of electron-withdrawing substituents, and thus lead to a positive value for $\rho(K_m)$, perhaps of the order of $\rho = 1.1$–2.2 as has been observed for the *O*-protonation of benzoic acids (35), benzamides (36), benzaldehydes (37), and acetophenones (38). The only interpretation of $\rho(K_m) = -0.53$ that is immediately apparent is in terms of some weak dipolar interaction. The coordination of the terminal carboxylate group to the metal ion is not ruled out by these observations, however, since this group is sufficiently far removed from the phenyl ring of the benzoic acid moiety, that it is effectively insulated from the electronic effects of substituents in this ring.

Deviations from the σ-Correlations

The above linear free energy correlations of k_{cat}, K_m, and k_{cat}/K_m with σ may be used to predict that $k_{cat} = 15\ min^{-1}$, $K_m = 7 \times 10^{-3}\ M$, and $k_{cat}/K_m = 2 \times 10^3\ M^{-1}\ min^{-1}$ for the unsubstituted benzoic acid ester (**1**: X = H). Clearly, if such parameters are applicable, the failure to observe enzymic hydrolysis of this ester may reflect pronounced substrate inhibition becoming important at much lower substrate concentrations than for any of the *para*-substituted benzoate esters that were investigated.

[4]See later discussion for our definition of "specific" and "nonspecific" substrates for this enzyme.

Such an interpretation would indicate a strong dependence of substrate inhibition on the presence of a *para*-substituent.

On the other hand, the lack of observable hydrolysis of the unsubstituted ester could also be attributed to predominant nonproductive binding of this substrate. If nonproductive binding is represented in terms of the following simple scheme:

$$E + S \underset{}{\overset{K_S}{\rightleftharpoons}} E.S \xrightarrow{k_2} E + P_1 + P_2$$

$$E + S \overset{K_S^{NP}}{\rightleftharpoons} E.S_{NP}$$

then, $k_{cat} = k_2/(1 + K_S/K_S^{NP})$ and $K_m = K_S/(1 + K_S/K_S^{NP})$. Thus, the observed k_{cat} and K_m values are both predicted to be reduced by the presence of nonproductive binding modes for the substrate. For $K_S^{NP} \ll K_S$, k_{cat} may become so small as to be unobservable. An obvious mode of nonproductive binding for this benzoic acid ester would involve "wrong-way" binding, in which the phenyl ring of the acid moiety enters the hydrophobic pocket which is believed (3, 4) to accommodate the alcohol hydrophobic side chain in the productive binding mode.

The linear correlations in Fig. 3 make it unlikely that nonproductive binding modes are important for these *para*-substituted esters, and again a remarkable substituent effect upon nonproductive binding seems to be present. This is in accord, however, with the very pronounced effect from the chlorine substituent in the binding of *O*-hippuryl-*p*-chloromandelic acid to this enzyme relative to the corresponding unsubstituted mandelic acid ester (39). Furthermore, we have also observed[5] that while the benzoate ion is a competitive inhibitor (K_i = 6 m*M* (21)) of esterase activity, the *p*-chloro-, *p*-nitro-, and *p*-methoxybenzoate ions are all noncompetitive inhibitors, and this again indicates a special effect from *para*-substituents in the binding of benzoic acid derivatives.

The complicated biphasic nature of the inhibition of the hydrolysis of *O*-hippuryl-L-3-phenyllactic acid by the unsubstituted benzoate ester is suggestive of the binding of more than one inhibitor species per enzyme molecule. This suggests that the observed lack of hydrolysis of this ester may in fact be due to a combination of the above postulated substrate inhibition (via formation of catalytically less active $E.S_2$ species, etc.) and nonproductive binding.

The *p-tert*-butyl benzoate ester has K_m approximately four-fold smaller than is predicted by the K_m–σ correlation and k_{cat} about seven-fold greater than predicted by the k_{cat}–σ correlation. The deviation of this substituent from the correlation lines cannot be ascribed solely to nonproductive binding, since such schemes predict diminished apparent k_{cat} values rather than the enhanced k_{cat} value observed in this case. If it is assumed that K_m reflects the dissociation constant for the E.S complex, it must be concluded that there is a special interaction of the *tert*-butyl group of this substrate with the enzyme, and that this interaction not only enhances binding but also results in an increased rate of hydrolysis. Similar interpretations have been suggested for other enzymic hydrolyses (*e.g.* chymotrypsin (18), emulsin (40)) in which the data for a *tert*-butyl substituent deviates significantly from linear free energy correlations defined by other substituents in a series of closely related substrates.

Consideration of pH–Rate Profiles

The observed p*H* dependence of k_{cat}/K_m for *O-p*-nitrobenzoylmandelic acid (Fig. 5) is similar to that previously observed for *O*-acetylmandelic acid (41). The estimated pK_a values for the operative enzymic dissociations in Fig. 5 are 6.95 and 7.9 which are comparable with the values observed (6.9 and 7.5) for *O*-acetylmandelic acid. The slightly higher value for the more basic ionization in the present case may be related to the presence of 5% dimethyl sulfoxide in the reaction solution. Although the p*H* dependence of k_{cat}/K_m for the *O*-hippuryl-3-phenyllactic acid (Fig. 7) is nominally of the same shape as for *O-p*-nitrobenzoylmandelic acid, the bell curve is much broader in this case and is best fitted by enzymic ionizations of pK_a = 5.8 and 9.3. These latter ionizations are similar to those reported for *O*-cinnamoyl-L-phenyllactic acid (6.6 and 9.4 (34)), for a series of *N*-protected oligodepsipeptide ester substrates (6.2 and ~9.1 (42)), and also a series of *N*-protected tripeptide substrates (6.1 and 9.1 (43)). Clearly, the ionization of pK_a ~ 7.5–7.9 in the *O*-acylmandelic acid substrates cannot be considered to arise from the same moiety as the ionization of pK_a ~ 9 that occurs in all the other ester and

[5]J. W. Bunting and C. D. Myers. Unpublished observations.

peptide substrates indicated above, and there is also some doubt whether the observed pK_a values ~6.9 and 6.1, respectively, in these two classes of substrates represent the ionization of the same enzymic acid. Furthermore, we feel that the differences between the p*H* dependences of k_{cat}/K_m for these two classes of substrates are sufficiently great that they can be conveniently used to define a distinction between specific (peptides and depsipeptides) and nonspecific (acetate and benzoate esters) substrates for this enzyme. It is interesting that according to this definition, *O*-cinnamoylphenyllactic acid appears to behave as a specific rather than a nonspecific substrate. We feel that this operational definition of specific and nonspecific substrates is more reasonable than the somewhat arbitrary distinctions that have been drawn in the past, and which have led to the classification of *O*-cinnamoyl-L-mandelic acid as a nonspecific substrate and *O*-cinnamoyl-L-3-phenyllactic acid as a specific substrate (44), and yet these two esters differ by only one methylene group.

The acidic groups which are generally considered to be most likely to be responsible for the ionizations discussed above are the phenolic OH of tyr-248, the γ-carboxyl of glu-270, a zinc-coordinated water molecule and possibly the imidazolium ions of his-69 and his-196 which appear to be coordinated to the zinc ion in the crystalline enzyme (3, 4). Auld and Vallee (45) favour the pK_a ~ 9 as being due to tyr-248 since the thermodynamic parameters for this ionization are typical of phenolic ionizations, and furthermore, this pK_a value is the same within experimental error for the Co, Mn, and Zn metalloenzymes which tends to rule out its assignment to the ionization of a metal-coordinated water molecule. However, on the basis of studies with the *O*-acetyltyr-248 enzyme, Kaiser and co-workers (46) have concluded that the pK_a ~ 9 should be assigned to $M(OH)^+$ formation, and have then suggested that the pK_a ~ 7.5 observed with *O*-acetylmandelic acid as substrate is due to tyr-248.

An enzymic pK_a ~ 7.0 is also observed in the modification of glu-270 by two different reagents (5, 7). This presumably reflects the same ionization as the pK_a ~ 6.9 that is observed in the hydrolysis of *O*-*p*-nitrobenzoyl- and *O*-acetylmandelic acids. It is tempting, of course, to assign this ionization to the γ-carboxyl group of glu-270 but there is no direct evidence that this is the case. Certainly, modification of glu-270 abolishes both peptidase and esterase activities towards *specific* substrates (5–8), and suggests an important functional role for this amino acid in the enzymic catalysis, although hindrance to binding through steric effects in the modified enzyme cannot be excluded.

Although it is clear that the same two acidic groups on the enzyme control the overall enzymic activity (k_{cat}/K_m) of the hydrolysis of both *O*-hippuryl-L-3-phenyllactic acid and related peptide substrates, the observed p*H* dependence of k_{cat} for this ester is different from that observed for peptide substrates (43). In fact, k_{cat} for this ester is remarkably p*H* independent over the range p*H* 5–10 (Fig. 7). For peptide substrates, k_{cat} is dependent on a pK_a ~ 6.1 in the acidic portion of this same range, but is not influenced by the group of pK_a ~ 9 (43). Clearly it is not possible to assign the pK_a ~ 6.1 to the γ-carboxyl of glu-270 and still claim a role for the conjugate base of this side chain in the hydrolysis of *both* these peptides and this ester. The p*H* dependence of k_{cat} for *O*-hippuryl-L-3-phenyllactic acid suggests, in fact, that *if* the conjugate base of the glu-270 γ-carboxyl group is involved in the hydrolysis of this ester, it must have a pK_a < 5. In fact, a pK_a ~ 4 for glu-270, typical of a "normal" glutamic acid γ-carboxyl group, is consistent with this assumption.

In the most recently proposed mechanisms for peptide hydrolysis by carboxypeptidase A (15), the phenolic OH of tyr-248 plays the role of a general-acid catalyst in the protonation of the amine leaving group. If such a mechanism is accepted, k_{cat} for peptide hydrolysis should clearly decrease upon deprotonation of tyr-248; therefore, pK_a ~ 9 cannot be assigned to tyr-248 on the basis of the current proposals for the mechanism of peptide hydrolysis, and if tyr-248 does act in this way as a general-acid catalyst, it must have pK_a > 10.

Adoption of the suggestion (46) that the pK_a ~ 9 which controls substrate binding to the free enzyme is due to ionization of the hydrated metal ion seems consistent with the observation that this pK_a does not seem to be present in the catalytically active E.S complex for specific ester and peptide substrates. Coordination of the free carboxylate ion or the carbonyl oxygen of the hydrolyzable bond to the metal ion would be expected to suppress this ionization in the E.S complex.

It is clear from the above discussion that at present it is not possible to rationalize all of the observed p*H*–rate profiles for ester and amide substrates of carboxypeptidase A in terms of simple catalytic roles for the amino acid side chains which are in the near vicinity of the zinc ion. The differences observed between specific and nonspecific ester substrates even further complicate the situation, and it is fair to say that not one of the ionization constants that have been observed to be catalytically important can be assigned with any certainty at present. The crystallographic investigation of the binding of glycyl-L-tyrosine indicates that the tyr-248 side chain undergoes a major movement upon binding this ligand (2–4). This leads one to wonder whether the binding of good substrates in solution may not involve other major conformational changes in the enzyme, and if such is the case, it is possible that one or more of the observed ionization constants may arise from an acidic group that is somewhat removed from the active site, but which controls an important conformational change.

A change in rate-determining step between formation and breakdown of a tetrahedral intermediate and/or acyl-enzyme is another possible factor which may be responsible for some of the observed p*H*–rate complexities, if this rate-determining step is both p*H* and substrate dependent. If such a factor is operative, an extremely detailed study of systematic substrate structural variations will be necessary before a complete analysis of the observed p*H*–rate profiles will be possible.

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