Mechanistic Studies of Carboxypeptidase Y. Kinetic Detection of an Acyl-Enzyme Intermediate in Trimethylacetate Esterase Action

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Abstract: Carboxypeptidase Y (protease C) from *Saccharomyces cerevisiae* possesses esterase activity toward the nonspecific substrates, aryl trimethylacetates. By means of stopped-flow spectrophotometry, the burst kinetics observed for 4-nitrophenyl trimethylacetate hydrolysis catalyzed by carboxypeptidase Y have been studied in detail. The results are quantitatively consistent with the intermediacy of a physical complex between enzyme and substrate followed by an acyl enzyme, trimethylacetyl-carboxypeptidase Y. Acylation rate constants (k_2/K_s) show a bell-shaped pH dependence on ionizations of pK_{app} = 5.94 and 7.92. Deacylation rate constants (k_3) show a sigmoidal dependence on an ionization of a group of pK_{app} = 5.1. This group is noncrucial as the rate constant in acidic media (0.99×10^{-2} s^{-1}) is 41% of the maximal rate constant in alkaline media (2.42×10^{-2} s^{-1}). In agreement with the acyl-enzyme hypothesis, $k_{cat.}$ values for a series of aryl-substituted trimethylacetates are independent of leaving group. The mechanism of action and site of acylation of carboxypeptidase Y are discussed in terms of the extent of catalysis in both acylation and deacylation and the natures of the catalytic groups involved.

Carboxypeptidase Y, otherwise known as protease C, was first isolated from yeast in 1967 by Hata et al.[1] In contrast to the pancreatic carboxypeptidases, A and B, it is a relatively nonspecific C-terminal exopeptidase. Carboxypeptidase Y apparently has no metal ion requirement and is not inactivated by chelating agents such as EDTA.[2] Its broad specificity and high stability to heat, pH, and denaturing agents[3] have led to its use in protein and peptide sequencing studies.[4] The Edman sequencing technique often encounters difficulties with the last half dozen or so residues (at the C-terminal fragment of the original protein) and it is just these residues for which a broad-ranging carboxypeptidase is most appropriate.

In addition to its protease and (presumably associated) peptidase activity, carboxypeptidase Y exhibits esterase activity toward substrates such as *N*-acetyltyrosine ethyl ester (ATEE) and even to 4-nitrophenyl acetate.[5,6] This nonspecific esterase activity prompted us to experiment with simple ester substrates as convenient tools for mechanistic investigation of carboxypeptidase Y. As will be described, the approach has culminated in our detection of two intermediates in the esterase action of carboxypeptidase Y, a Michaelis complex and a covalent acyl-enzyme intermediate. In addition, we are able to report the basis of the first active-site titration procedure for carboxypeptidase Y.

Experimental Section

Materials. Carboxypeptidase Y was prepared from Fleischmann compressed bakers' yeast as described by Hayashi et al.[4] The specific activities of the preparations were usually between 90 and 100 units using ATEE as substrate.

Trimethylacetate esters were synthesized by a nonaqueous Schotten–Baumann procedure described elsewhere.[7] 4-Nitrophenyl acetate was obtained from Eastman Kodak. *N*-Acetyl-L-tyrosine ethyl ester monohydrate was obtained from Aldrich Chemical Co. (mp 80 °C uncor). *N*-Carbobenzyloxy-α-L-glutamyl-L-tyrosine was purchased from Fox Chemical Co. Acetonitrile was either redistilled Aldrich Spectral Grade or, if of reagent grade, was redistilled twice by the method of O'Donnell et al.[8] before use. Buffer solutions were prepared from reagent grade chemicals to give an ionic strength of 0.10, unless otherwise stated.

Kinetic Methods. Kinetics were measured either by means of conventional, uv–visible spectrophotometry or using stopped-flow techniques. Conventional spectrophotometry was carried out on a Gilford-Beckman DU model or on a Cary 15 model spectrophotometer, each of which was fitted with a thermostated cell compartment regulated to 25.00 ± 0.01 °C. Stopped-flow measurements were made, when half-lives of reactions were less than ~2 s, on a Durrum-Gibson stopped-flow spectrophotometer with a thermostated syringe compartment. All studies were made at 25 °C and, consequently, solutions in cuvettes and syringes were preequilibrated for 5 min at this temperature.

For steady-state measurements on a conventional instrument the following procedure was generally adopted for determinations of the zero-order portions of the reaction time courses. Substrate (usually 50 μl of an acetonitrile stock solution, along with sufficient acetonitrile to give the desired final acetonitrile level) was equilibrated in 3.0 ml of the appropriate buffer. Reaction was initiated by addition of a suitable volume of enzyme solution (20–100 μl) with simultaneous commencement of a timer. As the chart recorder was switched on, the timer was stopped giving an accurate value for the mixing time (usually 5–7 s). Around neutrality the extinction coefficient of the products from reaction of 4-nitrophenyl trimethylacetate and carboxypeptidase Y is very sensitive to pH as the pK_a of 4-nitrophenol is 7.04.[9] Consequently, for any given pH, the value of the extinction change during reaction was measured by complete hydrolysis of a known concentration of substrate in the appropriate buffer and noting the absorbance change. From the results of this procedure the extinction changes were calculated. In cases where spontaneous hydrolysis of the substrate was significant (pH >8) inverse addition was adopted to obtain the absorbance change for complete hydrolysis, i.e., enzyme and buffer were equilibrated together in the cuvette, a baseline was recorded, and substrate was added on the flattened tip of a Teflon stirring rod. The substrates in this study have zero extinction coefficients at 400 nm, the wavelength used above pH 7. The cuvettes used had path lengths of 10 mm.

Stopped-flow studies were effected using the following techniques. Stock solutions of enzyme were prepared in degassed buffer to have twice the final enzyme concentration desired in the reaction cell. Similarly, substrate solutions were prepared to give substrate and acetonitrile concentrations twice those required in the mixing chamber. As 4-nitrophenyl trimethylacetate is relatively insoluble at the low concentrations of acetonitrile adopted, such substrate solutions were prepared by careful addition of degassed buffered solution to a concentrated stock solution of ester in acetonitrile. Careful checks for precipitation were made and such substrate solutions were prepared immediately before use. Enzyme and substrate solutions were loaded into the drive syringes, equilibrated at 25 °C and then rapidly mixed in equal volumes. The change in absorbance with time was recorded, and stored, on a type RM 564 storage oscilloscope (Tektronix). (In the later studies of this work data were collected in digital form by means of a Biomation Waveform Model 805 recorder.) Usually three to four runs, made under identical conditions, were superimposed upon one another on the oscilloscope screen and then photographed. As the stopped-flow unit was fitted with a differential amplifier the data were recorded directly in absorbance units.

Generally, pH measurements were made before and after reactions, the latter being carried out either within the reaction cuvette using

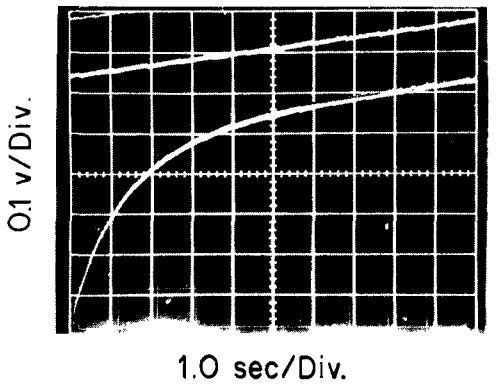


Figure 1. Stopped-flow photograph of burst kinetics observed with carboxypeptidase Y and 4-nitrophenyl trimethylacetate at pH 7.45, in the presence of 5.0% (v/v) acetonitrile. The ordinate represents 0.1 V per major division and the abscissa 1 s per major division (three runs overlapped).

a Thomas combination pH on the combined effluents from the reaction vessel for a series of runs in a given buffer in the case of stopped-flow studies. The values of pH before and after reaction differed by less than 0.04 pH unit and postreaction values were used in calculations. All pH measurements were made on a Beckman Research Model pH meter or a Radiometer Model PHM 4c pH meter, each of which was standardized against the appropriate Fisher standard buffer solution before use. As the maximum amount of acetonitrile was low (not more than 4% v/v), it was assumed[9] that this had a negligible effect on the electrode reading. All water used in this study was distilled in glass and subsequently deionized.

Above pH 6.05, kinetics for 4-nitrophenyl trimethylacetate hydrolyses were studied at 400 nm; at pH 6.05 and lower, the best wavelength was determined to be 330 nm by repetitive spectral scanning of a reacting mixture. Substrate stock concentrations were checked by complete hydrolysis of a sample in 0.1 M sodium hydroxide solution using an extinction coefficient of 18 000 at 400 nm.[9]

Approximate enzyme concentrations were measured using values of $A_{280\,nm}$ (1%) = 15 and a molecular weight of 61 000 daltons.[10] However, it will be shown, in the section on treatment of kinetic data, that it is unnecessary to know the absolute enzyme concentration for determination of k_3 as long as certain assumptions are valid (viz., $S_0 \gg K_m$, $k_2 \gg k_3$) and that, under the conditions employed, the pre-steady-state rate constants obtained are independent of the enzyme concentration.

Calculations and least-squares analyses of data were performed on a Hewlett-Packard Model 9100-A programmable calculator using an unweighted least-squares program for the treatment of Lineweaver–Burk data for acylation. Best fits of pH profiles were obtained using a nonlinear regression program with an IBM Model 370 computer written by B. A. Blumenstein of Emory University.

Enzyme Activity Assay Methods. The esterase activity of carboxypeptidase Y was routinely determined with ATEE as described by Hayashi et al.;[11] a Radiometer Titrator 11 with Titrigraph SBR 2c and a Radiometer pH meter 25 scale expander were used. Titrations were carried out at pH 8.0 with 0.02 N NaOH and the aid of a Radiometer Autoburette ABU 11. The reaction vessel was kept at 25 °C by circulating water from a constant temperature bath. Before each assay session the alkaline solution was standardized using a solution made with crystalline potassium biphthalate. Proteolytic activity of the enzyme was tested using *N*-carbobenzyloxy-α-L-glutamyl-L-tyrosine by the procedure of Hayashi et al.[4]

Results

"Burst" Kinetics Treatment. When yeast carboxypeptidase Y was allowed to react with excess 4-nitrophenyl trimethylacetate in sodium phosphate buffer (pH 7.45) containing 5.0% (v/v) acetonitrile, a rapid burst of 4-nitrophenol could be observed by measurement at 400 nm on the stopped-flow spectrophotometer; the burst was followed by a slower, zero-order release of 4-nitrophenol as shown in Figure 1. Similar burst kinetics have been reported with this substrate under comparable conditions with the endopeptidases, α-chymotrypsin[12] and elastase.[13]

The results were interpreted in the former case in terms of a scheme whereby free enzyme (E) and substrate (S) rapidly and reversibly form a physical Michaelis complex (ES) with a dissociation constant, K_s. A chemical step (rate constant k_2) converts this complex into the first product (P_1) and a covalent acyl enzyme (ES′), which then deacylates (k_3) to regenerate free enzyme and form the second product (P_2). Equation 1 summarizes this mechanism.

$$E + S \overset{K_s}{\rightleftharpoons} ES \xrightarrow{k_2} ES' + P_1 \xrightarrow{k_3} E + P_2 \qquad (1)$$

The full kinetic treatment of eq 1 has already been provided for α-chymotrypsin[12,14,15] and we need therefore only quote the results of that analysis. Under substrate in excess over enzyme conditions, the change in concentration of P_1 (here 4-nitrophenolate ion) as a function of time[12] is given by eq 2.

$$P_1 = At + \pi(1 - e^{-bt}) \qquad (2)$$

$$A = k_{cat.} \cdot E_0 \cdot S_0/(K_m + S_0) \qquad (3)$$

$$b = [(k_2 + k_3)S_0 + k_3 \cdot K_s]/(K_s + S_0) \qquad (4)$$

$$\pi = E_0[k_2/(k_2 + k_3)]^2/(1 + K_m/S_0)^2 \qquad (5)$$

and

$$k_{cat.} = k_2 \cdot k_3/(k_2 + k_3) \qquad K_m = K_s \cdot [k_3/(k_2 + k_3)] \qquad (6)$$

After a time ($>5/b$),[16] eq 2 reduces to eq 7 and the increase in P_1 with time is linear, with π as the intercept at $t = 0$ and A as the slope. This corresponds to the zero-order portion visible on Figure 1.

$$P_1 = At + \pi \qquad (7)$$

In contrast, at very low values of t, we can write eq 8

$$P_1 = At + \pi e^{-bt} \qquad (8)$$

and subtraction of eq 7 from eq 8 will allow determination of b by a semilogarithmic plot, i.e., treatment as a first-order equation. Under conditions where $S_0 \gg E_0$ and $k_3 \cdot K_s \ll (k_2 + k_3)S_0$ we can reduce eq 4 to

$$b = [(k_2 + k_3)S_0]/(S_0 + K_s) \qquad (9)$$

from which we see that the observed apparent first-order rate constant b should be independent of E_0 and show saturation as S_0 is increased. Further, taking reciprocals of eq 9 and carrying out a Lineweaver–Burk type plot allows separation of K_s and $(k_2 + k_3)$ if b is measured at several values of S_0.

Steady-State Kinetics of Carboxypeptidase Y with 4-Nitrophenyl Trimethylacetate. Equation 3 indicates that the zero-order slope A will equal $k_{cat.} \cdot E_0$ when $S_0 \gg K_m$. Table I shows that from pH 5.3 to 7.8 this situation holds, as A is sensibly independent of S_0.[17] Further, as $k_2 \gg k_3$ (this will be shown later to be the case over the above pH range) we can reduce eq 5 to πE_0. Thus, when $S_0 \gg K_m$ and $k_2 \gg k_3$

$$k_3 = A/\pi \qquad (10)$$

Equation 10 indicates that k_3 may be evaluated as the ratio of the zero-order slope to the burst in any internally consistent units and that absolute values of extinction coefficient need not be known. In essence, one is performing an active-site titration each time to obtain the enzyme concentration for measurement of k_3. Full details of a formalized active-site titration procedure will be published elsewhere.[28]

pH Profile for k_3. Using the procedure outlined in the previous section to measure k_3 (the ratio of the zero-order slope, A, to the burst, π), we have obtained values of k_3 over a wide range and these are collected in Table II. Figure 2 shows a plot of the pH profile for k_3. It is notable that k_3 depends on the ionization of a group of $pK_a = 5.1$, but that the overall change

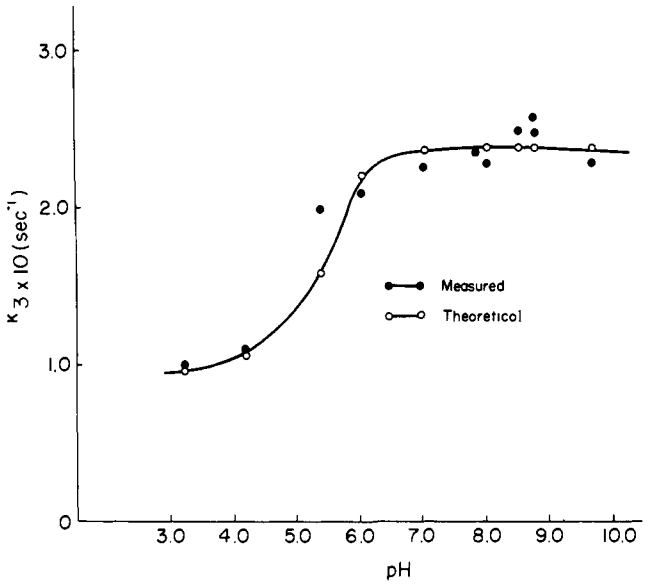


Figure 2. pH profile for deacylation (k_3) of trimethylacetyl-carboxypeptidase Y at 25 °C. Points are experimental (Table II); line is theoretical for an ionizing group of $pK_{app} = 5.1$ with limiting rate constants of 0.99×10^{-2} and 2.42×10^{-2} s^{-1} at high and low pH, respectively.

Table I. Effect of Substrate Concentration on Steady-State Parameters during Carboxypeptidase Y Catalyzed Hydrolysis of 4-Nitrophenyl Trimethylacetate[a]

pH	$S_0 \times 10^5$, M	Zero-order slope $\times 10^8$, M s^{-1}
7.84[b]	10.44	2.02
	6.96	2.02
	3.46	1.97
7.04[c]	10.63	3.94 ± 0.08
	8.51	4.28 ± 0.11
	7.09	4.09 ± 0.02
	5.67	3.89 ± 0.07
	4.96	3.91 ± 0.03
5.30[d]	10.44	3.38
	6.96	1.37
	3.48	1.38

[a] Measured at 25 °C and ionic strength 0.1, 2.4% acetonitrile. [b] $E_0 = 1.82 \times 10^{-6}$ M, sodium phosphate buffer used. [c] $E_0 = 1.22 \times 10^{-6}$ M, sodium phosphate buffer used. [d] $E_0 = 1.22 \times 10^{-6}$ M, sodium acetate buffer used.

in k_3 over the pH range is only 2.3-fold. Further, deacylation is still rapid in acidic solution; the limiting rate constants of deacylation (k_3) in acidic and basic solution are 0.99×10^{-2} and 2.42×10^{-2} s^{-1}, respectively.

Presteady-State Kinetics of Carboxypeptidase Y with 4-Nitrophenyl Trimethylacetate. We have seen from eq 9 in the section on "burst kinetics" that, in the presteady state, a first-order plot of the exponential portion of the curve (corrected for steady-state turnover of the enzyme reaction and spontaneous substrate hydrolysis) yields a rate constant b. Measurement of b as a function of substrate concentration, with $S_0 \gg E_0$, allows separation of K_s and $(k_2 + k_3)$ by use of a Lineweaver-Burk type plot of $1/b$ vs. $1/S_0$.[18] An example of such a double-reciprocal plot is given in Figure 3. As seen from eq 4 or 9 when $S_0 \gg E_0$ we expect to find that b is independent of the initial enzyme concentration when S_0 is held constant. At pH 6.23 with an initial substrate concentration of 7.53×10^{-5} M and initial enzyme concentrations between 0.8 and 3.2×10^{-6} M, the value of b was 0.454 ± 0.003 s^{-1}.

In Table III, we have collected presteady-state data at various pH's for carboxypeptidase Y catalyzed hydrolysis of 4-nitrophenyl trimethylacetate. Figure 4 shows the pH profile

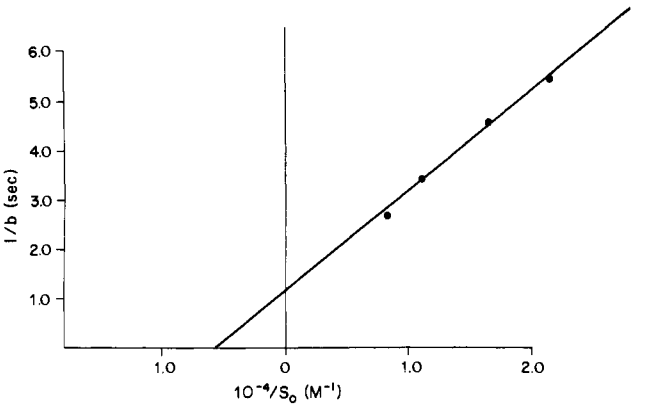


Figure 3. Double-reciprocal plot of data for the presteady-state (acylation) reaction of 4-nitrophenyl trimethylacetate with carboxypeptidase Y at pH 5.36 in sodium acetate buffer ($\mu = 0.1$, acetonitrile 5.0% v/v). Points plotted are the averages of at least three consecutive runs; the correlation coefficient for the line, obtained by unweighted linear regression analysis, was 0.997. Enzyme concentration was 1.153×10^{-6} M.

Table II. Steady-State pH Profile Data for Carboxypeptidase Y Catalyzed Hydrolysis of 4-Nitrophenyl Trimethylacetate[a]

pH	Buffer[b]	$k_3 \times 10^2$, s^{-1}
3.24	Formate	0.99
4.21	Acetate	1.09
5.42	Acetate	2.00
6.05	Phosphate	2.10
7.09	Phosphate	2.26
7.88	Phosphate	2.35
8.04	Phosphate	2.28
8.52	Tris	2.49
8.82	Tris	2.57
8.85	Barbital	2.48
9.73	Carbonate	2.28

[a] Each value of k_3 is determined from the zero-order slopes at three different enzyme concentrations; all measurements were made using a single enzyme preparation and on 1 day. [b] Ionic strength 0.1 except at pH 8.85 where it was 0.05.

Table III. Data for pH Profile of Presteady-State Rate Constants for Carboxypeptidase Y Catalyzed Hydrolysis of 4-Nitrophenyl Trimethylacetate[a]

pH	k_2/K_s[b] $\times 10^{-4}$, M^{-1} s^{-1}	k_2,[b] s^{-1}	K_s[b] $\times 10^4$, M^{-1}
4.12	0.087 ± 0.024		
4.68	0.200 ± 0.022		
5.10	0.574 ± 0.058	0.43 ± 0.05	0.752
5.46	0.363 ± 0.003		
5.69	0.405 ± 0.027	Not separable	
6.23	0.835 ± 0.012	2.01 ± 0.13	2.41
6.85	1.20 ± 0.10	1.48 ± 0.20	1.24
7.16	1.07 ± 0.06	1.71 ± 0.19	1.64
7.45	0.793 ± 0.002	4.15 ± 0.92	5.24
7.84	0.814 ± 0.002	Not separable	

[a] Enzyme preparation number 13 was used. Acetonitrile level was 5% (v/v) with an ionic strength of 0.1. [b] From a least-squares regression analysis of a double reciprocal plot of $1/b$ (observed presteady-state rate constant) vs. $1/S_0$.

for k_2/K_s under these conditions. The profile is bell-shaped depending on groups with pK_{app} of 5.94 ± 0.27 and 7.92 ± 0.44. The precisions of the separated constants k_2 and K_s do not warrant the individual drawing of pH profiles of these parameters, however.

Steady-State Kinetics of Carboxypeptidase Y with Aryl-Substituted Trimethylacetates. Burst kinetics are not only observed with 4-nitrophenyl trimethylacetate but also with other aryl-substituted trimethylacetates.[19] We have studied

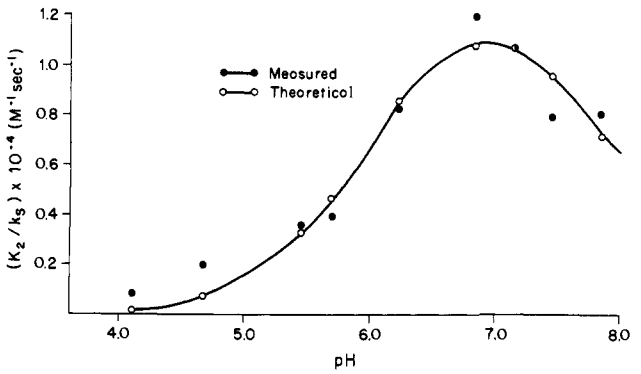


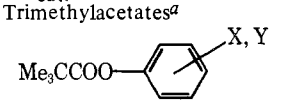
Figure 4. Acylation (k_2/K_s) pH profile for presteady-state reaction of 4-nitrophenyl trimethylacetate with carboxypeptidase Y. The line is theoretical for a bell-shaped profile based on ionization of two groups of $pK_{app} = 5.94 \pm 0.27$ and 7.92 ± 0.44 and was obtained by best computer fit to the data using the kinetic scheme

$$EH_2 \overset{K_{a1}}{\rightleftharpoons} EH + H^+ \overset{K_{a2}}{\rightleftharpoons} E + 2H^+$$

inactive ↓ inactive

products

Table IV. Values of $k_{cat.}$ in Carboxypeptidase Y Catalyzed Hydrolysis of Aryl Trimethylacetates[a]



X,Y	$\Delta\epsilon_{rxn}$	λ, nm	$k_{cat.}$,[b] $s^{-1} \times 10^2$	pK of product phenol
2,4-$(NO_2)_2$	1.14×10^4	400	2.75 ± 0.10	3.96[c]
2-Cl, 4-NO_2	1.58×10^4	400	2.72 ± 0.10	5.45[d]
4-NO_2	1.04×10^4	400	2.92 ± 0.07	7.04[e]

[a] Measured in pH 7.35 phosphate buffer (0.1 M) in the presence of 3.2% (v/v) acetonitrile at 25 °C. [b] The values of $k_{cat.}$ were obtained using zero-order slope = $k_{cat.} \cdot E_0$ and are based on E_0 values obtained from $A_{280} = 15$ and molecular weight of 61 000; the active site titration procedure was not developed at the time of these experiments. All values were obtained on the same day using three different concentrations of a single enzyme preparation, for maximum internal consistency. [c] "CRC Handbook of Chemistry and Physics," 50th ed, 1969, Table D120. [d] V. E. Bower and R. A. Robinson, *J. Phys. Chem.*, 64, 1078 (1960). [e] See ref 9.

the carboxypeptidase Y catalyzed cleavage of 2-chloro-4-nitrophenyl and 2,4-dinitrophenyl trimethylacetates under the steady-state conditions already described for the 4-nitro ester with $S_0 \gg E_0$. The values of $k_{cat.}$ obtained are collected in Table IV. These results have been commented on briefly in a preliminary communication.[19]

Effect of α-Toluenesulfonyl Fluoride on Burst Kinetics. The effects of α-toluenesulfonyl fluoride on the burst kinetics observed in the carboxypeptidase Y catalyzed hydrolysis of 4-nitrophenyl trimethylacetate are reported in Table V. Both the burst and turnover reactions are inhibited by incubation, indicating that the k_2 and/or K_s step(s) are blocked by this inhibitor. Full kinetic details of the tosyl fluoride inhibition of carboxypeptidase Y will be reported in due course.

Discussion

To preface discussion of the mechanistic implications of the results described above, it is advisable to show that carboxypeptidase Y is indeed a catalyst for trimethylacetate hydrolysis and, as far as possible, to quantitate the extent and efficiency of catalysis.

Further, as we have used, in essence, an active-site titration based on the trimethylacetate reaction, the active site we titrate

Table V. Effect of α-Toluenesulfonyl Fluoride on the Carboxypeptidase Y Catalyzed Cleavage of 4-Nitrophenyl Trimethylacetate[a]

$S_0 \times 10^5$, M	$I_0 \times 10^5$, M	Incubation time, min	Burst $\times 10^6$, M	Zero-order slope $\times 10^8$, M s^{-1}
9.64	0	5[b]	0.922	2.99
9.64	6.55	5[b]	0.183	0.207
6.47	0	30	1.59	1.56
6.47	0	60	1.14	1.66
6.47	6.60	30	0.083	0
6.47	6.60	60	0.111	0

[a] Incubations with the stated concentration of inhibitor were carried out at 25 °C in pH 7.04 sodium phosphate buffer ($\mu = 0.1$) with an enzyme concentration of 1.33×10^{-6} M (based on $A_{280} = 15$, mol wt 61 000). Unless otherwise stated the incubation mixture contained 2.4% of acetonitrile. Assays were performed by addition of substrate in acetonitrile to the incubation mixture and recording the absorbance at 400 nm. [b] Incubation mixtures contained 3.02% (v/v) of acetonitrile.

Table VI. Kinetic Parameters for Catalyses of Aryl Trimethylacetates. Hydrolysis by Carboxypeptidase Y and Other Catalysts

Parameter	Me_3CCOX, X = $-OC_6H_4$-p-NO_2	X = $-OC_6H_5$	X = $-OCOC(CH_3)_3$
k_2, s^{-1} [a]	0.81		
K_S, M[a]	2.21×10^{-5}		
k_3, s^{-1} [b]	2.2×10^{-2}		
k_2/K_S, M^{-1} s^{-1} [a]	3.85×10^4		
k_{HO^-}, M^{-1} s^{-1} [d]	0.923[d]	0.13	7.95×10^{-5}[c]
k_{H_2O}, s^{-1}			
$k_{imid.}$, M^{-1} s^{-1}	3.04×10^{-2}	1×10^{-5}[d]	

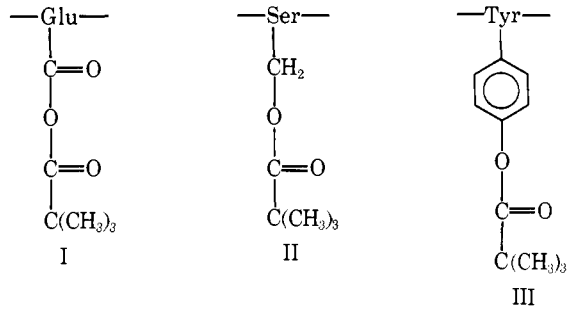
[a] Values from Table IV. [b] pH 7–9, 2.4% acetonitrile, $\mu = 0.1$ (see Table III). [c] C. A. Bunton and J. H. Fendler, *J. Org. Chem.*, **30**, 1365 (1965). [d] Predicted from a linear free-energy relationship in K. T. Douglas, Y. Nakagawa and E. T. Kaiser, unpublished results.

with this relatively unnatural substrate must be the same as, or bear a simple stoichiometric relationship to, the active site implied in the specific esterase activities currently used in assay procedures (e.g., ATEE). Blocking, by α-toluenesulfonyl fluoride, of the esterase activities of this enzyme toward ATEE[3] and 4-nitrophenyl trimethylacetate (Table V) indicates that, unless there is considerable protein flexibility, the sites of specific and nonspecific esterase activity are identical or very close stereochemically. We have also found that Hg(II) ions inhibit the trimethylacetate reaction, but little can be said about this at present as we have so far only studied effects in the steady state.[20] A report of the effect of organomercurials on the esterase action of carboxypeptidase Y has appeared recently.[21]

Extent of Catalysis. In a parallel investigation, we have studied intensively the reactivity of the trimethylacetyl center, observing the rate effects of changes in both nucleophile and leaving groups.[7] Attack of hydroxide ion and imidazole on trimethylacetates is nucleophilic for all esters studied.[7,22] In Table VI we have collected some selected rate constants for enzymatic (carboxypeptidase Y) and nonenzymatic reactions of various trimethylacetate derivatives.

The most obvious comparison, for 4-nitrophenyl trimethylacetate, is that the apparent bimolecular rate constant or specificity constant[23] for the enzymatic acylation reaction (k_2/K_s) is some 4×10^4 times larger than k_{HO^-} and approximately 10^6 times greater than $k_{imid.}$ Acylation of carboxypeptidase Y by this simple ester substrate is highly catalyzed even compared to catalysis by very efficient nucleophiles such

as hydroxide ion or imidazole. We can look at catalysis of deacylation in some detail. The active-site sequence in carboxypeptidase Y is -Glu-Ser*-Tyr-, wherein Ser* is the residue containing the radioactivity after [^{32}P]diisopropyl phosphofluoridate incorporation and work-up.[24] In principle, the diisopropylphosphofluoridate reaction can be explained by initial phosphorylation of any member of this particular trio of residues (see later). During esterase action, glutamic acid, serine, or tyrosine could be acylated giving the following possible acyl-enzyme partial structures.



I II III

For α-chymotrypsin with 4-nitrophenyl trimethylacetate as substrate at pH 8.17 in Tris-HCl buffer (μ = 0.06 M, 1.8% acetonitrile, 25 °C), $k_2 = 0.37\ s^{-1}$, $K_s = 1.6 \times 10^{-3}$ M, and $k_3 = 1.3 \times 10^{-4}\ s^{-1}$.[12] Comparison of K_s indicates that binding of this ester to carboxypeptidase Y is more powerful than to α-chymotrypsin although k_2 values are comparable.[25] However, trimethylacetyl carboxypeptidase Y deacylates about a hundred times more rapidly than does trimethylacetyl-α-chymotrypsin. Such ready deacylation suggests a highly labile acyl enzyme, e.g., possibly I or III rather than the alkyl ester II. Acyl enzyme I is an analogue of trimethylacetic anhydride and, indeed, k_3 is only some 250 times greater than the spontaneous water-catalyzed rate of hydrolysis for trimethylacetic anhydride (see Table VI); the rate difference would be even smaller if the mixed anhydride of propionic and trimethylacetic acid was compared. However, were the acyl enzyme a tyrosine derivative, fair comparison might be with phenyl trimethylacetate for which the observed rate constant at pH 7 would be of the order of $10^{-8}\ s^{-1}$, indicating considerable catalytic enhancement in deacylation. Catalysis would be even greater if the rate of a nonactivated alkyl trimethylacetate were to be compared, i.e., if a serine acyl enzyme were involved.

The isolation of a peptide containing radioactivity in a sequence -Gly-Ser*-Tyr- could have been caused not only by direct phosphorylation of a reactive serine residue but also by attack of the phosphoryl fluoride on the glutamic acid (to give a mixed phosphocarboxylic anhydride) or on tyrosine [to produce an activated (phenyl)phosphate ester]. Either of these species might well be expected to undergo ready intrapeptide phosphoryl transfer during work-up for sequencing and analysis. Such a sequence is especially prone to intramolecular acyl transfer to serine, as the phosphoserine ester produced is the most stable product. This possibility, hitherto overlooked, should be borne in mind when considering other enzymes which have been shown to contain the -Glu-Ser*-Tyr- active site sequence, e.g., the carboxypeptidase from bean leaves, plant phaseolin.[26]

Acyl-Enzyme Hypothesis for Carboxypeptidase Y. Throughout this paper, we have adopted the kinetic analysis appropriate for an acyl-enzyme mechanism in the enzymatic catalysis of aryl trimethylacetate hydrolysis by carboxypeptidase Y. Such an approach was prompted by the observation of biphasic, burst kinetics (with respect to 4-nitrophenol release from 4-nitrophenyl trimethylacetate under conditions of substrate in excess over enzyme). This kinetic model quantitatively explains all of our results to date.

As further support of the acyl-enzyme hypothesis we note the constancy of $k_{cat.}$ values for a series of substituted aryl trimethylacetates (see Table IV) of constant acyl structure, which supports the view that all of these esters hydrolyze under the catalytic influence of carboxypeptidase Y with a common rate-determining step under steady-state conditions. Such a step is readily explained as the deacylation of trimethylacetyl-carboxypeptidase Y. The nucleophilic reactions of aryl-substituted trimethylacetates show considerable dependence on the nature of the leaving group (as measured, for example, by the pK_a of the leaving group conjugate acid). Thus, in reactions with hydroxide ion and imidazole as nucleophiles, respectively, the following linear free-energy relationships are obeyed.[7]

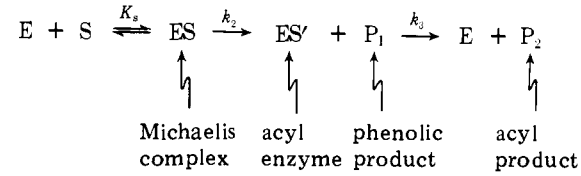
$$\log k_{HO^-} = 1.030 - 0.97\ pK_{L.G.} \qquad (r = 0.987) \quad (11)$$

$$\log k_{imid} = 9.95 - 1.17\ pK_{L.G.} \qquad (r = 0.982) \quad (12)$$

Such observations contrast strongly with the independence that $k_{cat.}$ shows of leaving group in the reactions of carboxypeptidase Y.

We recognize that biphasic kinetics of the type observed merely indicate a mechanism in which a change in rate-determining step is possible.[27] Although the high stability of carboxypeptidase Y to denaturation by SDS[28] and urea[4] argues against major conformational flexibility of the enzyme, it cannot be taken as firm ground from which to discuss minor changes in tertiary structure, such as those which might be involved in product desorption. However, the 4-nitrophenyl (and several other aryl) ester(s) of *trans*-cinnamic acid are good substrates for carboxypeptidase Y. The phenolic portion of the product is released prior to, and faster than, the cinnamate moiety in the carboxypeptidase Y catalyzed hydrolysis of 4-nitrophenyl *trans*-cinnamate. Furthermore, we have detected kinetically an intermediate (as yet, unisolated) in the enzymatic hydrolysis of 4-methoxyphenyl *trans*-cinnamate; presumably this is cinnamoyl-carboxypeptidase Y. These results will be duly reported in full, but a preliminary account of them has been presented.[29] There is ample literature precedent for acyl-enzyme mechanisms in the action of endopeptidases (e.g., α-chymotrypsin,[30] papain[31]), but these are the first such kinetic demonstrations for an intracellular exopeptidase.

We have observed saturation with respect to change in the presteady-state rate constant with increased substrate concentration. Consequently, the minimal kinetic scheme for *this* esterase action of carboxypeptidase Y involves two distinct intermediates, i.e., a physical Michaelis complex prior to formation of the covalent acyl enzyme.



The techniques used do not permit detection of intermediates occurring before ES on the reaction pathway; i.e., K_s may refer to the overall equilibrium constant for a series of Michaelis complexes in rapid equilibrium with each other.

Details of the Catalytic Mechanism. To this point in the discussion the overriding impression one would have of carboxypeptidase Y mechanism is that it is distinctly similar to that of α-chymotrypsin. One can summarize this similarity in terms of the inhibitions by diisopropylphosphorofluoridate and tosyl fluoride and the similar esterase activities toward trimethylacetates and cinnamates. In addition, one can quote the acyl-enzyme schemes adopted and the apparently similar substrate specificities, e.g., toward ATTE, and α-benzoyl-

L-tyrosine *p*-nitroanilide.[5,6,32] However, looking slightly more deeply into the situation shows that the similarity ends. Deacylation of trimethylacetyl-α-chymotrypsin depends crucially on the state of ionization of a functional group of $pK_a = 6.8$.[32] In marked contrast, deacylation of trimethylacetyl-carboxypeptidase Y depends, *noncrucially,* on an ionization of $pK_{app} \sim 5.1$. In addition, bovine pancreatic trypsin inhibitor does not inhibit the activity of carboxypeptidase Y.[32]

The overall low sensitivity to pH of $k_{cat.}$, especially between pH 7 and 10, is a situation reminiscent of the pH dependency of $k_{cat.}$ values for the reaction of carboxypeptidase A with some esters, e.g., *O*-hippuryl-L-β-phenyllactate.[34] We have already discussed the unusually high rate of deacylation (k_3) for trimethylacetyl-carboxypeptidase Y (compared, for example, to α-chymotrypsin[25]). Should an anhydride-based acyl enzyme be involved one might expect its hydrolysis to proceed rapidly over a wide pH range with essentially a pH-independent behavior.

It is tempting to invoke ionization of a glutamic acid residue to explain the pH dependency of k_3 for carboxypeptidase Y, especially as the active-site sequence is -Glu-Ser-Tyr-. The noncrucial nature of this group may mean that the ionization of the acidic species involved simply exerts an electrostatic effect on the active site. However, other explanations are possible. The group of $pK_{app} = 5.1$ may exert a mild conformational control of the active site. It should be noted that if the sequence -Glu-Ser-Tyr- is part of an α-helical segment, the glutamic acid and serine side chains will not be very close stereochemically;[35] i.e., under such circumstances the group of $pK = 5.1$ is unlikely to be this glutamic acid exerting an electrostatic effect. However, if the active-site sequence is part of a nonhelical segment, or if a conformational change occurs on ionization of this glutamic acid, it could give rise to the pH dependency observed of k_3. The noncrucial ionization seen at $pK = 5.1$ might be ascribed to a carboxyl group, a *different* one than is involved in anhydride acyl-enzyme formation, if such occurs.

The comments made previously on the ambiguity of the DFP reaction with carboxypeptidase Y make assignment of a site of acylation difficult, especially as we have little data on the variation of presteady-state parameters with pH. Hayashi et al.[5,6] report dependence of $k_{cat.}/K_m$ values on ionizations of $pK_{app} = 4.4$ and 5.8 for peptides while, for ester substrates, $k_{cat.}/K_m$ values depend on ionizations of $pK_{app} = 5.8$ and 8.9. Although it is possible that different sites participate in the esterase and peptidase activities, one cannot precisely interpret $k_{cat.}/K_m$ values for such specific substrates because of the complexity of the kinetic scheme. For example, if an acyl-enzyme mechanism is adopted by specific substrates it cannot yet be said whether acylation and/or deacylation is rate determining for peptidase and esterase activities. Conformational as well as kinetic complications must also be considered. A similar view must be taken of the solvent deuterium isotope studies recently carried out on carboxypeptidase Y[6]—again effects on $k_{cat.}$ are complex and difficult to interpret until the rate-determining step for the reaction chosen is clearly defined. After the publication of our previous communications[19,25] on the trimethylacetate-esterase activity of carboxypeptidase Y, subsequent workers have failed to observe similar burst kinetics.[6] However, at the low enzyme concentrations of their study the predicted burst would have been <0.004 absorbance units.

In conclusion, we can say that the nonspecific esterase action of carboxypeptidase Y probably involves an acyl-enzyme intermediate on the reaction pathway after formation of a physical Michaelis complex. Even with the trimethylacetyl group, deacylation is extremely rapid, making it likely that for specific substrates and less deactivated acyl groups (e.g., acetyl) the rate-determining step will be largely acylation. In agreement with this we have failed to observe burst kinetics with 4-nitrophenyl acetate even with enzyme concentrations at which such burst would be readily detected.[36] For the smaller and less lipophilic acetyl group, deacylation is presumably no longer significantly slower than acylation under experimentally accessible conditions.

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(17) The validity of the assumption that $S_0 > K_m$ can also be shown by considering the effect of varying S_0 on the value of π predicted by eq 5. For the constraint $S_0 \gg K_m$, π must be independent of S_0, and, for example, at pH 7.04 in sodium phosphate buffer in the presence of 2.4% acetonitrile (v/v), π was found to have the value $2.19 \pm 0.15 \times 10^{-6}$ M in the substrate concentration range of $(4.96\text{–}10.63) \times 10^{-5}$ M for five different values of S_0.
(18) This is true as long as $(k_2 + k_3)S_0 \gg k_3K_s$. In one experiment we found that when $S_0 = 1.25 \times 10^{-4}$ M, $b = 0.93\ s^{-1}$. No saturation was observed at this substrate concentration and we may rather overestimate K_s as 10^{-2} M (Table III indicates that where measurable K_s is much lower than this value). Taking $k_{cat.}$ as $2 \times 10^{-2}\ s^{-1}$ (see Table II), k_3 cannot be greater than this value. Equation 4 rearranges to
$$b(K_s + S_0) = (k_2 + k_3)S_0 + k_3K_s$$
allowing us to estimate k_2 as $0.71 \times 10^2\ s^{-1}$. Using this estimate to compare terms, we find that the k_3K_s term is negligible relative to the $(k_2 + k_3)S_0$ term, even with these gross overestimates of k_3 and K_s.
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