The Functional Tyrosyl Residues of Carboxypeptidase A. Nitration with Tetranitromethane*

James F. Riordan, Mordechai Sokolovsky,† and Bert L. Vallee

ABSTRACT: Nitration of carboxypeptidase A with tetranitromethane increases esterase activity to 180% of the control while reducing peptidase activity to less than 10%, under standard assay conditions. A fourfold molar excess of reagent at pH 8.0 for 30 min at 20° has been optimal for nitration. The inhibitor, β-phenylpropionate, protects against changes in activity due to nitration. Spectral and amino acid analyses demonstrate that the alterations in activity are due to the modification of a single tyrosyl residue. The kinetic parameters, K_m and V_{max}, of the nitro enzyme indicate that nitration affects both the catalytic and binding steps as well as substrate inhibition. On nitration the anomalous pH–rate profile of native carboxypeptidase changes, in part due to an effect on substrate inhibition. Nitrocarboxypeptidase is compared with azocarboxypeptidase (Sokolovsky, M., and Vallee, B. L. (1967), *Biochemistry 6*, 700). In the latter, also only one tyrosyl residue is coupled with diazo-1H-tetrazole, resulting in an increased esterase activity, but, in contrast to the nitro enzyme, in only a slight decrease of peptidase activity. A series of successive chemical modifications of carboxypeptidase suggest that nitration and azo coupling each affect only one, but different, tyrosyl residues which may, therefore, be functionally distinct. Minimally, these two tyrosyls, together with the zinc atom, appear essential to the catalytic activity of the enzyme. Kinetic and chemical modification studies are discussed in terms of the hypothesis postulating dual or multiple substrate binding loci previously proposed (Vallee, B. L., 152nd National Meeting of the American Chemical Society, New York, N. Y., Sept 1966, Abstract 83C).

Tetranitromethane has been shown to be a convenient reagent for the nitration of tyrosyl residues in proteins (Riordan *et al.*, 1966; Sokolovsky *et al.*, 1966). Nitration of carboxypeptidase A with TNM[1] modifies one tyrosyl residue and nearly doubles esterase activity while decreasing peptidase activity to less than 10% under standard assay conditions (Sokolovsky *et al.*, 1966; Riordan *et al.*, 1967). These changes can be prevented by the competitive inhibitor, β-phenylpropionate, suggesting that this tyrosyl residue is part of the active center of the enzyme. We have now examined the details of the nitration procedure and the enzymatic characteristics of the resultant nitroenzyme.

Chemical modifications had implicated tyrosyl residues in the function of carboxypeptidase previously. Thus, acetylation of the enzyme with either acetic anhydride or *N*-acetylimidazole increases esterase activity six- to sevenfold and markedly decreases peptidase activity (Simpson *et al.*, 1963; Riordan and Vallee, 1964). These changes in activities could be correlated with the acetylation of two active center tyrosyl residues. Succinylation, iodination, photooxidation, and several other reactions also alter tyrosyl residues with analogous catalytic consequences (Vallee, 1964). In these instances, however, the number of residues modified has not been examined. A limited molar excess of 5-diazo-1H-tetrazole leads to coupling of a single tyrosyl residue in carboxypeptidase (Sokolovsky and Vallee, 1967). Esterase activity increases twofold but, in contrast to nitration, peptidase activity decreases only slightly. A series of successive chemical modifications of carboxypeptidase suggest that nitration and azo coupling affect different residues, each of which is functionally distinct. Minimally, these two tyrosyls, together with the zinc atom, appear essential to the catalytic activity of carboxypeptidase.

Materials

Carboxypeptidase A prepared by the method of Anson (1937) was purchased as an aqueous crystal suspension from the Worthington Biochemical Corp. The crystals were washed three times with deionized distilled water and dissolved in 3 M NaCl to give stock solutions of about 10^{-3} M which were stable for at least 1 month at 4°. The proteolytic coefficient of the native enzyme (*C*) was 35–40 measured at 0°, while the esterase activity (*k*) was 7.5–8.0 × 10^3 moles/min per mole of enzyme measured at 25°. Tetranitromethane was obtained from Aldrich Chemical Co. and was used without purification. All other chemicals were of the

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[1] Abbreviations used: TNM, tetranitromethane; CGP, carbobenzoxyglycyl-L-phenylalanine; HPLA, hippuryl-*dl*-β-phenyllactic acid; DHT, 5-diazo-1H-tetrazole.

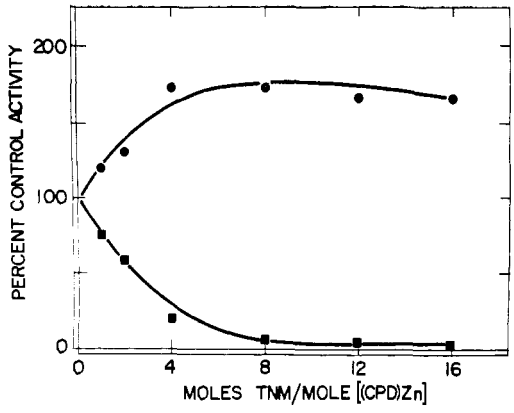

FIGURE 1: Effects of variation of the molar excess of TNM on the esterase (●) and peptidase (■) activities of carboxypeptidase.

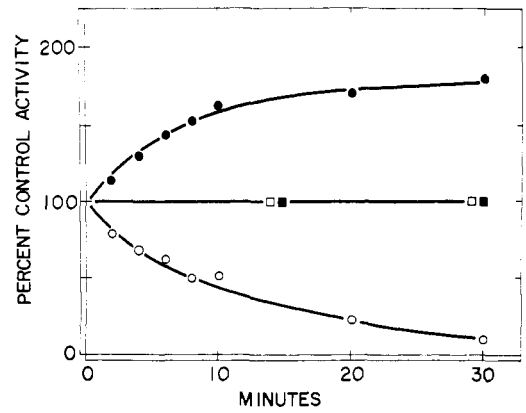

FIGURE 2: Progression of changes in esterase (closed symbols) and peptidase (open symbols) activities during nitration of carboxypeptidase (10 mg/ml) with a fourfold molar excess of TNM in the presence (□, ■) and absence (○, ●) of 0.1 M β-phenylpropionate.

best grade available. Buffers were extracted with 0.1% dithizone in carbon tetrachloride to avoid contamination by adventitious metal ions.

Methods

Peptidase activity was determined using 0.02 M carbobenzoxyglycyl-L-phenylalanine (CGP) (Cyclo Chemical Corp.) in 0.02 M Veronal–1 M NaCl (pH 7.5) (Coleman and Vallee, 1960) and *esterase activity* was measured using 0.01 M hippuryl-*dl*-β-phenyllactate (HPLA) in 0.005 M Tris–0.2 M NaCl (pH 7.5) by pH-Stat titration (Simpson *et al.*, 1963). A spectrophotometric esterase assay (McClure *et al.*, 1964) was employed for substrate concentrations below 0.001 M.

Protein concentrations were determined by absorbance at 278 mμ using a molar absorptivity of 6.42×10^4 M^{-1} cm^{-1} (Simpson *et al.*, 1963). A Zeiss PMQII spectrophotometer was used for measurements of absorbance at single wavelengths and a Cary 15 MS recording spectrophotometer was employed for determination of absorption spectra. The pH of solutions was measured on a Radiometer Model 22 pH meter equipped with a 2021 C glass–calomel combination electrode. Zinc analyses were performed by atomic absorption spectrometry (Fuwa and Vallee, 1963).

Amino acid analyses were performed with a Spinco 120 B amino acid analyzer utilizing the chromatographic procedures of Spackman *et al.* (1958). Acid hydrolysis was carried out in sealed, evacuated tubes with 6 N HCl at 105° for 22 hr. Tryptophan was determined on unhydrolyzed protein by the method of Spies and Chambers (1949).

Nitration was performed by addition of a 1 or 10% solution of TNM in ethanol to a solution of carboxypeptidase (10 mg/ml) in 0.05 M Tris–2 M NaCl (pH 8.0) for 45 min at room temperature (20°) (Riordan *et al.*, 1967). The reaction was terminated by passing the mixture through a Bio-Gel-P4 column. Acetylcarboxypeptidase was prepared using *N*-acetylimidazole as previously described (Simpson *et al.*, 1963). Iodocarboxypeptidase was prepared according to Simpson and Vallee (1966) with an eightfold molar excess of iodine monochloride or a 25-fold molar excess of iodine–KI. Azosuccinylcarboxypeptidase was prepared by first succinylating the native enzyme with a 48-fold molar excess of succinic anhydride (dialyzing for 36 hr to allow deacylation of tyrosyl residues) (Riordan and Vallee, 1964), followed by a twofold molar excess of DHT (Sokolovsky and Vallee, 1967). *O*-Acetyltyrosyl residues were determined using the neutral hydroxamate procedure of Balls and Wood (1956).

Results

Nitration Conditions. Nitration of carboxypeptidase with 5 μl (42 μmoles) of TNM per ml of enzyme solution (10^{-4} M) at pH 8.0 and 20° almost doubles esterase activity and reduces peptidase activity to less than 10% of the control within 1 hr. Under these conditions the solubility of TNM in aqueous buffers is only about 5×10^{-4} M, suggesting that much lower molar excesses of reagent might suffice to produce the same changes in activity. Consequently, nitration was carried out with different amounts of TNM using a dilute ethanolic solution of the reagent (Figure 1). A fourfold molar excess of reagent increases esterase activity maximally; no further change occurs with up to a 16-fold molar excess. With a fourfold molar excess peptidase activity falls sharply to about 15% of the control and gradually decreases with increasing amounts of reagent until it is less than 5% after reacting with a 16-fold molar excess. These changes in peptidase activity are not limited to CGP, the particular substrate used for routine assays. Little or no activity was observed when hippuryl-, acetyl-, chloroacetyl-, or formylphenylalanine were employed.

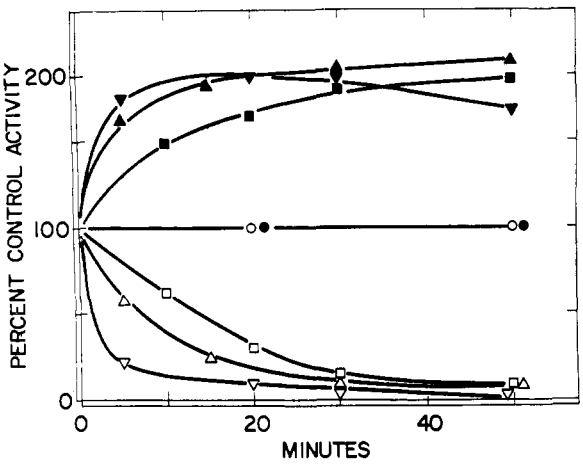

FIGURE 3: Effects of variation of the pH at which nitration is performed on the esterase (closed symbols) and peptidase (open symbols) activities of carboxypeptidase: pH 6.0 (○, ●); pH 7.5 (□, ■); pH 8.0 (△, ▲); pH 9.0 (▽, ▼).

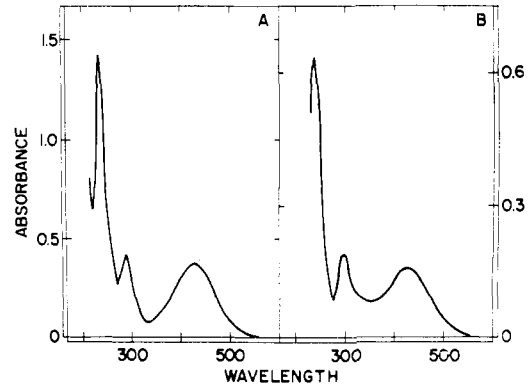

FIGURE 4: Absorption spectral studies. (A) The absorption spectrum of *N*-acetyl-3-nitrotyrosine minus *N*-acetyltyrosine both at 1×10^{-4} M. (B) The absorption spectrum of nitrocarboxypeptidase minus carboxypeptidase both at 1.76×10^{-5} M. Both spectra were obtained in 0.05 M Tris–1 M NaCl, pH 8.0, at room temperature.

The time course of the nitration reaction was followed with a 4-fold molar excess of TNM at pH 8.0 (Figure 2). Esterase activity increases to 180% of the control within 30 min. Simultaneously peptidase activity falls to less than 10% over the same time interval. Neither activity changes further when the reaction is allowed to proceed for up to 3 hr. Nitration in the presence of 0.1 M β-phenylpropionate, a competitive inhibitor of the enzyme, is without effect on enzymatic activities (Figure 2). Enzyme nitrated in the presence or absence of β-phenylpropionate and then dialyzed *vs.* 0.05 M Tris–1 M NaCl (pH 8.0) overnight contained 1.04 g-atoms of zinc/mole.

Nitration of carboxypeptidase is markedly dependent on pH (Figure 3). The rate at which peptidase activity is lost increases progressively as the pH at which nitration is performed is raised from 7.5 to 9.0. At pH 6.0, activity does not change, even after 3 hr. The rate at which esterase activity increases depends on pH in an analogous fashion, but the final, maximal activity which can be obtained is virtually the same at pH 7.5 and 9.0.

A given molar excess of TNM alters activities dependent on the amount of protein being nitrated. The increase in esterase or decrease in peptidase activity becomes less as the protein concentration decreases below 5 mg/ml. When between 5 and 20 mg of protein/ml are employed, however, the functional consequences of nitration appear to be independent of concentration.

The nature of the buffer can also influence the modification reaction. The rates of nitration in Tris or Veronal buffer do not differ, but they are significantly reduced in borate or phosphate. These last two buffer salts inhibit carboxypeptidase, an observation which may be pertinent.

Quantitation of the Nitration Reaction. Previous studies with amino acids and peptides showed that TNM nitrates tyrosyl[2] and oxidizes cysteinyl residues at pH 8.0 and 20°. Since carboxypeptidase does not contain free sulfhydryl groups, primary attention was directed to tyrosine. At pH 8.0 the absorption spectrum of *N*-acetyl-3-nitrotyrosine has a maximum at 428 mμ which is also present in the absorption spectrum of nitrated carboxypeptidase. The difference spectrum of nitrated minus native carboxypeptidase (Figure 4b) is virtually identical with that of 3-nitrotyrosine minus tyrosine (Figure 4a) and provides evidence for the existence of nitrotyrosyl residues in the modified protein.

The molar absorptivity at 428 mμ of the phenolate species of 3-nitrotyrosine is 4200. On protonation the absorbance maximum shifts to 360 mμ, and ϵ_{360} is 2790. The degree of nitration can be determined from the absorbance either at 428 mμ or at the nitrophenol–nitrophenolate isosbestic point at 381 mμ, ϵ_{381} 2200. The nitration of tyrosyl residues of carboxypeptidase with a fourfold molar excess of TNM was followed by removing aliquots at different times and measuring the absorbance both at 381 and 428 mμ subsequent to the removal of nitroform by gel filtration. Approximately 1.2 moles of nitrotyrosine/mole of protein are introduced after 45 min (Table I). If the nitration reaction is carried out in the presence of 0.1 M β-phenylpropionate, only 0.3 tyrosyl residue is nitrated.

About 90% of the decrease in peptidase activity observed on nitration of carboxypeptidase with molar excesses of TNM ranging from 1 to 24 correlates with

[2] While spectral and pH–titration data indicate a stoichiometric conversion of tyrosine to 3-nitrotyrosine (Sokolovsky *et al.*, 1966), the recovery of 3-nitrotyrosine has varied from 40 to 60%. This discrepancy, not observed with tyrosyl residues in peptides and proteins, is still under investigation.

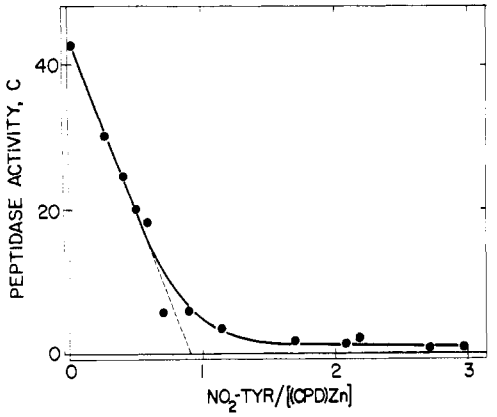

FIGURE 5: Correlation of the peptidase activity of nitrocarboxypeptidase with the number of tyrosyl residues nitrated. Molar excesses of TNM ranging from 1 to 24 were employed to obtain the various degrees of nitration. Nitrotyrosyl content was determined by spectral analysis.

the modification of only one tyrosyl residue (Figure 5). On nitration of up to three tyrosyl residues, activity falls to less than 5%. These data are not corrected for the number of tyrosyl residues nitrated in the presence of β-phenylpropionate by the same molar excess of TNM. Determinations of the nitrotyrosyl content of nitrocarboxypeptidase by amino acid analyses and spectral measurements are in close agreement (Table II). Moreover, these data indicate that TNM did not destroy residues other than tyrosine. The tryptophan content of the enzyme during the course of nitration was determined separately by a colorimetric method and did not change under these conditions over a period of 1 hr.

Nitration of Chemically Modified Carboxypeptidase.

TABLE I: Nitration[a] of Carboxypeptidase with TNM in the Presence and Absence of β-Phenylpropionate: Nitrotyrosyl Content.[b]

Time (min)	NO_2-Tyr/Mole	
	Protected[c]	Unprotected
5		0.25
10	0.14	0.62
20	0.21	0.88
30	0.3	1.25
45	0.3	1.2

[a] Nitration was carried out with a fourfold molar excess of TNM, 20°, 0.05 M Tris–1 M NaCl, pH 8.0. [b] Determined both spectrally and by amino acid analyses (Sokolovsky *et al.*, 1966). [c] In the presence of 0.1 M β-phenylpropionate.

TABLE II: Amino Acid Composition of Carboxypeptidase[a] Nitrated with a Fourfold Molar Excess of TNM.

Amino Acid	Native	Nitro
Lys	15.1	14.7
His	7.6	7.5
Arg	10.0	10.1
Asp	26.2	26.6
Thr	22.5	22.7
Ser	28.3	28.1
Glu	22.8	24.9
Pro	10.0	9.8
Gly	21.9	22.1
Ala	19	19
Val	14.2	14.8
Met	2.7	2.6
Ile	16.0	17.6
Leu	22.3	23.8
Tyr	17.9	16.5
Phe	14.5	15.1
NO_2-Tyr		0.92
Trp[b]	7.9	7.7

[a] Results of single analyses expressed as moles of amino acid per mole of enzyme and calculated on the basis of 19 alanines/mole. [b] Colorimetric determination with *p*-dimethylaminobenzaldehyde (Spies and Chambers, 1949).

Acetylation, iodination, and coupling with diazo-1H-tetrazole (DHT) all increase the esterase activity of carboxypeptidase due to modification of active center tyrosyl residues. The first two also markedly reduce peptidase activity, but a limited molar excess of DHT alters peptidase activity only slightly. Each of these modified carboxypeptidases was nitrated with TNM under the standard conditions at pH 8.0 for 45 min (Figure 6). The activities of acetylcarboxypeptidase are not affected by this treatment. Esterase activity remains at about 600% relative to the native enzyme while peptidase activity is barely measurable. Removal of TNM from acetylcarboxypeptidase by gel filtration followed by reaction with 1 M hydroxylamine for 10 min restores activities characteristic of the native enzyme.

Nitration of iodocarboxypeptidase progressively decreases esterase activity from 550 to about 200%, a value characteristic of nitrocarboxypeptidase. The very low peptidase activity of this sample is unchanged during the 45-min reaction time.

Azosuccinylcarboxypeptidase containing 1.2 moles of monoazotyrosine/mole of enzyme had an esterase activity of 180% and a peptidase activity of 95% relative to the native enzyme. Nitration of this derivative *did not affect its esterase activity but did decrease its peptidase activity* to that characteristic of nitrocarboxypeptidase. Spectral analysis of the product revealed

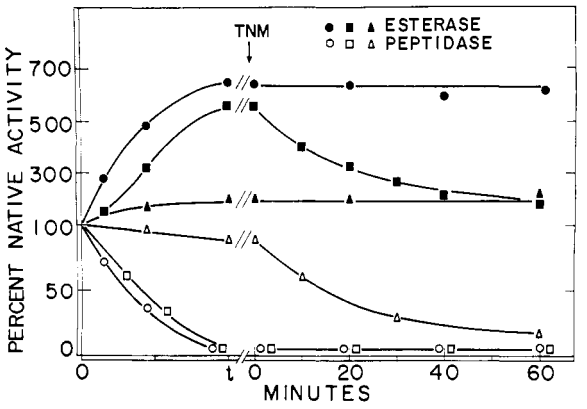

FIGURE 6: Progression of esterase (closed symbols) and peptidase (open symbols) activities on reaction of acetyl- (●, ○), iodo- (■, □)- and azo-1H-tetrazolylcarboxypeptidase (▲, △) with a fourfold molar excess of TNM at pH 8.0, 20°. The left-hand part of the figure indicates the time course for the changes in activities on acetylation with acetylimidazole (circles), iodination with iodine in KI (squares), and coupling with DHT (triangles). The time scale is 30 min for acetylation, 150 min for iodination, and 30 min for coupling. The right-hand part of the figure indicates the changes in activities subsequent to the addition of TNM.

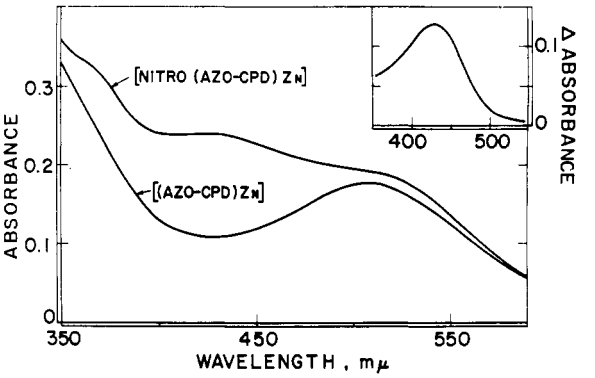

FIGURE 7: The absorption spectra of azocarboxypeptidase and nitroazocarboxypeptidase in 0.05 M Tris–1 M NaCl (pH 8.0), both at 3.1×10^{-5} M. The inset represents the difference spectrum of nitroazocarboxypeptidase minus azocarboxypeptidase.

the presence of only *one* azotyrosyl and *one* nitrotyrosyl residue each per mole (Figure 7).

If the order of the chemical modifications is reversed, *i.e.*, nitration precedes acetylation, iodination, or azo coupling, the activities do not change. During and subsequent to the exposure of the enzyme to these other reagents its esterase and peptidase activities remain at the levels characteristic of nitrocarboxypeptidase. The acetylated nitrocarboxypeptidase contains 3.1 *O*-acetyltyrosyl groups/mole as measured by the neutral hydroxamate procedure (Balls and Wood, 1956). Acetylation in the presence of 0.1 M β-phenylpropionate, however, results in the formation of only 2.25 *O*-acetyl groups, a difference of 0.85.

Kinetic Properties of Nitrocarboxypeptidase. The carboxypeptidase-catalyzed hydrolysis of HPLA is known to be inhibited by substrate as well as products (McClure *et al.*, 1964; Bender *et al.*, 1965). Maximal hydrolysis occurs at a substrate concentration of 7×10^{-4} M (Figure 8). The kinetic constants derived from initial rate data obtained at this and lower concentrations are $K_{m\ app} = 5.1 \times 10^{-5}$ M and $V_{max} = 2.8 \times 10^{4}$ min^{-1} (McClure *et al.*, 1964) or $K_{m\ app} = 8.8 \times 10^{-5}$ M and $V_{max} = 3.5 \times 10^{4}$ min^{-1} (Bender *et al.*, 1965). Nitration increases the $K_{m\ app}$ for HPLA hydrolysis approximately 30-fold to 1.9×10^{-3} M and correspondingly shifts substrate inhibition to higher concentrations. V_{max} for nitrocarboxypeptidase is 1.6×10^{4} min^{-1}, about one-half that of the native enzyme. The twofold increase in activity observed on nitration appears to result from an increase in $K_{m\ app}$ and the shift in substrate inhibition which combine to generate a new activity maximum at 0.01 M HPLA, the concentration used for routine assays.

Nitration of carboxypeptidase also alters the esterase pH–rate profile (Figure 9). The activity of the native enzyme, measured with 0.01 M HPLA, increases as the pH is increased from 5.5 to 7.0 and then remains almost constant up to pH 9.5. At higher pH values, activity increases sharply to reach an apparant maximum at pH 10.5. However, if the pH dependence of esterase activity is measured at 0.0005 M HPLA, the profile is bell-shaped with an optimum between pH 7.5 and 8.0. The activity of nitrocarboxypeptidase has a bell-shaped profile when measured at 0.01 M HPLA with a broad optimum between pH 7.5 and 8.5, similar to that observed for iodocarboxypeptidase (Simpson and Vallee, 1966).

Discussion

The functional importance of tyrosyl residues in carboxypeptidase was recognized largely as a consequence of chemical modifications using *N*-acetylimidazole, acetic and succinic anhydrides, iodine, and several other reagents, all of which increase esterase and decrease peptidase activities (Vallee, 1964). Nitration with TNM, known to modify tyrosine and tyrosyl peptides (Riordan *et al.*, 1966; Sokolovsky *et al.*, 1966), produces similar functional effects. Under standard conditions of assay the esterase activity of nitrocarboxypeptidase is almost twice that of the unmodified control while its peptidase activity is reduced to less than 10%. As with previous modifications, the functional consequences of nitration can be prevented by β-phenylpropionate and other inhibitors. By analogy, the changes in carboxypeptidase activity provide first indications that nitration modifies tyrosyl residues of this enzyme. This is supported by the pH dependence of the nitration of carboxypeptidase (Figure 3) which

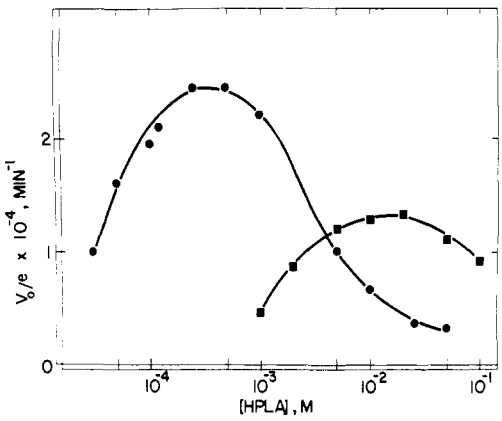

FIGURE 8: The substrate concentration–velocity profiles for the esterase activities of (●) native and (■) nitrocarboxypeptidase measured with hippuryl-*dl*-phenyllactate.

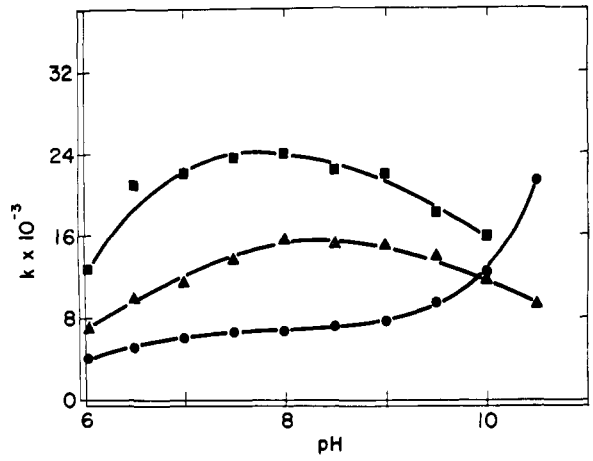

FIGURE 9: The esterase pH–rate profiles for native (●, ■) and nitro (▲) carboxypeptidase. The substrate concentration was 0.01 M for (●) and (▲) and was 0.0005 M for (■). Activities were measured either by pH-Stat titration (●, ▲) or by spectrophotometry (■). The data above pH 9.0 have been corrected for spontaneous hydrolysis of substrate.

is similar to that observed with tyrosine and tyrosyl peptides (Riordan *et al.*, 1966).

Nitrotyrosyl residues can be determined qualitatively and quantitatively by virtue of their absorption at 428 mμ. By this criterion, 1.2 tyrosyl residues are modified when carboxypeptidase is exposed to a fourfold molar excess of TNM at pH 8.0 for 45 min. These conditions bring about changes in enzymatic activity, virtually maximal for this agent, suggesting that modification of only a single tyrosyl residue can account for the observed results. Thus, both by chemical and functional criteria, nitration of tyrosine by TNM would appear to be the most selective modification of carboxypeptidase observed thus far.

Amino acid analyses confirm the nitrotyrosyl content of carboxypeptidase derived from spectral analyses (Table II), and demonstrate further that treatment with TNM does not destroy other amino acids. In particular, the histidyl, lysyl, and methionyl contents of the native and nitro enzymes are identical.

The nitration of tyrosine and tyrosyl peptides can also be quantitated by measuring the increase in absorbance at 350 mμ owing to the formation of the trinitromethyl carbanion (nitroform), ϵ_{350} 14,400, or by titrating the protons released (Riordan *et al.*, 1966). However, these latter methods proved to be unsatisfactory for carboxypeptidase. Instead of reaching an equilibrium value, both the absorbance at 350 mμ and the release of protons increase continuously, suggesting that side-chain groups of the protein may act as general bases catalyzing the hydrolysis of TNM. Under these circumstances, the measurement of either nitroform or protons is not an accurate gauge of nitration.

Cysteine is the only other amino acid found to react with TNM so far (Riordan *et al.*, 1966; Sokolovsky *et al.*, 1966). However, carboxypeptidase does not contain free sulfhydryl groups and zinc is bound to the sole cysteinyl residue of the apoenzyme, the metal mercaptide being essential for activity. Since nitrocarboxypeptidase still contains 1 g-atom of zinc/mole and clearly retains esterase activity, it would appear that this residue is not affected. Moreover, even on removal of zinc from carboxypeptidase this SH group does not become accessible to TNM either at pH 8.0 where both cysteinyl and tyrosyl residues should react or at pH 6.0 where only the former react. The precise chemical identity of the atypical sulfur-containing amino acid residue, which does not appear to participate in the catalytic function of carboxypeptidase, is still unknown (Walsh *et al.*, 1962). While this group might conceivably be modified by TNM (Kawanami, 1964) this would not account for the changes in enzymatic activity nor for the protective effect of β-phenylpropionate (Figure 2).

No evidence for an interaction of TNM with tryptophanyl residues could be detected either in model compounds or in carboxypeptidase (Table II). It should be emphasized, however, that the lack of modification by this reagent of a particular amino acid residue in a given protein cannot exclude its reaction in other proteins owing to specifically favorable conditions provided by a suitable environment.

With larger molar excesses of TNM more than the one functional tyrosyl residue of carboxypeptidase can be nitrated. After four successive additions of a fourfold molar excess of TNM three tyrosyl residues per mole are nitrated though there are no additional functional changes (Figure 1). After eight additions of an eightfold molar excess, *i.e.*, a total of 64 moles of TNM/mole of enzyme, 6.7 residues/mole are modified, resulting in a slight decrease of esterase activity. Further additions of reagent under these conditions do not nitrate additional tyrosines. Thus, similar to

N-acetylimidazole (Riordan *et al.*, 1965), TNM may be a convenient reagent for determining "free" and "buried" tyrosyl residues in proteins.

Previous spectrophotometric titrations and reaction with *N*-acetylimidazole also indicated that seven of the 19 tyrosyl residues of carboxypeptidase are "free" while 12 are "buried." As judged both by protection with inhibitors and substrates and by the reversible changes of activities on acetylation and deacylation, two of the seven "free" tyrosyls apparently participate directly in biological activity while the other five do not. This suggests a further subdivision of the "free" tyrosines based on function as well as chemical reactivity. The deacylation of two tyrosyl residues correlates with the restoration of native activity and is more rapid than that of the remaining five. Nevertheless, though equivalent chemically, these two groups need not be equivalent functionally. Reaction with TNM indicates that, indeed, they are not, since one of the two (referred to hereafter as Tyr 1) can be nitrated selectively and differentiated from the other (*vide infra*). Spectrophotometric titrations demonstrate a possible chemical basis for the increased reactivity of Tyr 1 toward TNM, which may bear on its functional role. After nitration the phenolic hydroxyl group of this residue is unusually acidic. Addition of β-phenylpropionate to nitrocarboxypeptidase perturbs the nitrotyrosyl residue to "normalize" its pK either due to direct binding or to conformational changes. These data suggest that in the native enzyme this residue is activated, perhaps due to features of its immediate chemical environment (Riordan *et al.*, 1967).

Nitration differs from acetylation and iodination of carboxypeptidase in the molar excess of reagent required, the number of residues modified, and the activities of the resultant products. The maximal increase in esterase activity with TNM occurs with only a fourfold molar excess of reagent. In contrast, a 60-fold molar excess of acetylimidazole, a 48-fold molar excess of acetic anhydride, a 25-fold molar excess of iodine in KI, and a 7-fold molar excess of iodine monochloride is needed for the characteristic, maximal changes to be observed on modification with these reagents. Although spontaneous decomposition of the acylating agents may provide a partial explanation for these differences, acetylation and iodination affect several tyrosyl residues per mole of enzyme. Acetylation modified six to seven tyrosyls per mole and four to five in the presence of β-phenylpropionate. However, it has not been possible thus far to prepare and isolate a monoacetyl enzyme, the functional characteristics of which could either exhibit all of the changes observed on acetylation or be different, *e.g.*, analogous to those of the mononitro enzyme.

Iodination modifies five to six tyrosyl residues of carboxypeptidase. While β-phenylpropionate completely protects against the changes in activities, it prevents, at most, only 0.6 mole of tyrosine/mole of enzyme from being iodinated, as evidenced by spectral titration, amino acid analysis, and incorporation of ^{131}I (Simpson and Vallee, 1966), as well as paired labeling experiments Roholt *et al.*, 1965).[3] Thus on the basis of present data it cannot be determined that the enzymatic changes of iodocarboxypeptidase are due to the iodination of one or of two tyrosyl residues (Vallee, 1964; Simpson and Vallee, 1966).

A number of successive chemical modifications have been performed to discern if one or both tyrosyl residues have a function in the catalytic activity of carboxypeptidase. Treatment with TNM does not alter the activities of acetylcarboxypeptidase further nor does acetylation with *N*-acetylimidazole or acetic anhydride change those of nitrocarboxypeptidase. This is entirely consistent with studies on model compounds. Under the conditions here employed *O*-acetyltyrosine cannot be nitrated and the phenolic hydroxyl groups of nitrotyrosine cannot be acetylated. Further, iodination does not alter the activities of nitrocarboxypeptidase. This is consistent with earlier observations that the functional effects of iodination and acetylation are mutually exclusive (Simpson and Vallee, 1966).

It was, therefore, of interest that nitration of iodocarboxypeptidase reduces esterase activity from 29.7 to 12.2 $\times$ 10^3 moles/min while peptidase activity remains less than 5% of the native enzyme (Figure 6). Since iodination affects the active center tyrosyl residues (*vide supra*) nitration might have modified yet other residues. However, TNM was found to react very rapidly with both mono- and diiodotyrosine. Though the products of these reactions have not been identified as yet, it is possible that the nitronium ion displaces iodine and the conversion of iodotyrosyl to nitrotyrosyl residues would then explain the activity changes observed on nitration of iodocarboxypeptidase. Thus, it would appear that Tyr 1 is also among those tyrosines which are acetylated or iodinated.

The question whether or not the second tyrosyl residue (Tyr 2) is functional can be examined by means of DHT. Coupling with an eightfold molar excess of this reagent also alters only one tyrosyl residue, resulting in a twofold increase in esterase activity *but without concomitant loss of peptidase activity* (Sokolovsky and Vallee, 1967). Nitration with TNM does not change the esterase activity of monoazocarboxypeptidase *but reduces the peptidase activity* to less than 5%. Spectral analysis reveals that the product contains one azotyrosyl and one nitrotyrosyl residue each per mole (Figure 7). Hence, the loss of peptidase activity must be attributed to nitration of a second tyrosyl residue, not to the displacement of the azo group. In view of the specificity of nitration it would appear that DHT reacts with Try 2 and TNM with Tyr 1. Similar results have been obtained previously on acetylation or iodination of azocarboxypeptidase (Sokolovsky and Vallee, 1967). The evidence accumulated thus far indicates that the zinc atom and two tyrosyl residues are involved directly or indirectly in substrate binding and/or catal-

[3] NOTE ADDED IN PROOF. Roholt, O. A., and Pressman, D. (1967), *Proc. Natl. Acad. Sci. U. S. 58*, 280, have now reported the sequence of an active center peptide isolated from iodocarboxypeptidase to be Ile-Tyr-Glx-Ala.

ysis. Yet other residues may be found to participate, of course.

The magnitude of the increase in esterase activity appears to be determined more by the specific chemical characteristics of the modified residue than by its modification *per se*. Thus, acetyl-, iodo-, and nitrocarboxypeptidases differ in the rates at which they hydrolyze HPLA. On acetylation and iodination the esterase activity of the native enzyme increases six- to sevenfold but on nitration it increases only twofold when assayed with 0.01 M substrate. In view of the known substrate and product inhibition and product activation of carboxypeptidase, differences in the rates of ester hydrolysis cannot be interpreted unambiguously by comparison of values obtained at a single substrate concentration. These kinetic anomalies may be affected variously by the different chemical modifications, thereby giving rise to the different activities. On the other hand, the chemical characteristics of tyrosine, nitrotyrosine, and *O*-acetyltyrosine differ, of course; hence, there is no reason to expect identical kinetic properties of the native, nitro, and acetyl enzymes, particularly if the properties of the modified tyrosyl group are presumed to play a role in determining the final enzymatic behavior. However, structural and conformational changes of the modified enzymes, though not detected thus far by conventional means, cannot be ruled out both as the quantitative and the qualitative basis of the enzymatic activities.

The increased esterase activity of nitrocarboxypeptidase is not due to abolition of substrate inhibition. Rather, it is the result of an increase in K_m and a corresponding displacement of the substrate concentration range in which inhibition is manifested. These two effects generate a new activity maximum at about 0.01 M HPLA which is reflected as an apparent twofold increase in activity (Figure 8). Similarly, acetylation increases K_m and displaces substrate inhibition to virtually the same extent as nitration. Again maximal activity occurs at about 0.01 M HPLA. In this case, however, V_{max} is also increased, and, hence, an apparent sixfold activation is observed.

The anomalous esterase pH–rate profile of native carboxypeptidase may be pertinent to the role of tyrosyl residues in carboxypeptidase (Figure 9). The sharp increase in activity above pH 9.0 when assayed using 0.01 M HPLA could suggest either a specific role for hydroxide ions in the catalytic process or, alternatively, might reflect ionization of a group of a protein side chain, perhaps tyrosine. When assays are performed at a substrate concentration of 0.0005 M activity no longer increases above pH 9.0, and the profile becomes bell-shaped (Figure 9). The velocity *vs.* substrate concentration profile indicates marked substrate inhibition at 0.01 M HPLA, but not at 0.0005 M. Thus, the anomalous pH–rate profile would seem to be related closely to substrate inhibition, perhaps reflecting its release above pH 9.0 where peptidase activity decreases also. It is of interest that the pH–rate profiles of all the organically modified enzymes, including nitrocarboxypeptidase, are bell-shaped and their pH optima are virtually identical. Moreover, substrate inhibition is not discernible at 0.01 M. Since the pK's of the modified tyrosines differ in each case (Sokolovsky *et al.*, 1967) it appears unlikely that the pK of the phenolic hydroxyl group(s) of the active center tyrosine(s) would be the sole determinant for the pH dependence of ester hydrolysis.

It has been suggested (Vallee, 1966, 1967) that the anomalous kinetic behavior of carboxypeptidase toward acylamino acids, dipeptides, and esters results from binding of these substrates and their products to multiple loci on the enzyme.[4] Such multiple binding loci could arise in part from the fact that the enzyme is capable of acting upon larger substrates, *i.e.*, oligopeptides (Schechter and Berger, 1966) and polypeptides (Coombs and Vallee, 1966), as well as proteins. Hence, small substrates might not uniquely fit to complement the entire potential binding region. The enzyme might then accommodate a number of these small substrate molecules simultaneously, thereby leading to the anomalous kinetics. X-Ray crystallographic data indicate that certain dipeptides can bind to the enzyme in more than one manner (Lipscomb *et al.*, 1967).

Our view suggests that, on one hand, dipeptide and ester substrates may bind "correctly," resulting in a catalytically productive interaction. Alternatively, they could bind "incorrectly," yielding catalytically nonproductive complexes which could however be inhibitory. The proposed "dual" or "multiple loci" model postulates that nonidentical but overlapping binding of dipeptides and analogous esters could be one basis for much of the existent kinetic data.

The alterations in peptidase and esterase activities in response to different inorganic and organic modifications may encompass various combinations of effects on catalytic efficiency, substrate binding, and substrate inhibition. Data on acetylcarboxypeptidase, consistent with this hypothesis, have been presented (Vallee, 1967). Since nitration affects only one of the two tyrosyl residues implicated by acetylation, analogous studies on the nitro enzyme should allow the recognition of the role of this particular residue in substrate binding or catalysis.

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[4] Binding refers to the over-all process, including both formation of the enzyme–substrate complex, as seen in direct binding studies, or of any intermediate which can differ readily from these and dominate the kinetic results.

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