Iodocarboxypeptidase*

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ABSTRACT: Iodination of carboxypeptidase A either with iodine or iodine monochloride increases esterase activity up to five or six times the control value. Simultaneously peptidase activity is suppressed almost completely, similar to the results previously observed upon acetylation [Simpson, R. T., Riordan, J. F. and Vallee, B. L. (1963), *Biochemistry 2*, 616; Riordan, J. F., and Vallee, B. L. (1963), *Biochemistry 2*. 1460]. Iodination alters the kinetics of hippuryl β-*dl*-phenyllactate hydrolysis characteristic of the native enzyme. When present during iodination, β-phenylpropionate, a competitive inhibitor of the enzyme, prevents these alterations of enzymatic activity. The iodination procedure employed modifies tyrosyl residues. Iodination subsequent to acetylation of carboxypeptidase suggests that both of these procedures modify the same functional residues. The iodoenzyme is homogeneous by ultracentrifugation, and other physicochemical properties are also indistinguishable from those of the native protein.

Differences in the pH–rate profiles for ester hydrolysis of the native and iodoenzymes suggest a possible role for tyrosyl residues in esterase activity. The loss of peptidase activity is discussed in terms of alteration of groups involved in the binding of peptide substrates.

Native carboxypeptidase A hydrolyzes both synthetic peptides, *e.g.*, carbobenzoxyglycyl-L-phenylalanine, and esters, *e.g.*, hippuryl *dl*-β-phenyllactate.[1] Acetylation, iodination, or photooxidation markedly alter the dual specificity of the enzyme (Vallee *et al.*, 1963). When measured under standard assay conditions (*vide infra*), these chemically modified carboxypeptidases hydrolyze esters at rates considerably greater than that of the native enzyme, while their peptidase activity is virtually nonexistent. Since competitive inhibitors or substrates prevent these functional changes (Vallee *et al.*, 1963; Riordan and Vallee, 1964), it has appeared likely that the selective reagents interact with a limited number of residues at the active center of the enzyme.

The reactivity of the agents employed suggested early (Vallee *et al*, 1963) that tyrosyl or histidyl residues, or both, might be involved in the mechanism of action of carboxypeptidase. Previously we have shown that acetylimidazole and mono- and dicarboxylic acid anhydrides alter the activity of the enzyme by preferential acylation of two reactive tyrosyl residues at the active center (Simpson *et al.*, 1963; Riordan and Vallee, 1963, 1964). The present data are the results of investigations on carboxypeptidase modified either with iodine monochloride or with iodine. A preliminary report of these studies has appeared (Simpson and Vallee, 1963).

Experimental Section

Materials

Crystalline carboxypeptidase A prepared by the method of Anson (1937) was purchased from the Worthington Biochemical Co., Freehold, N. J., and was utilized for all studies except those involving metal removal. For these investigations, five times recrystallized carboxypeptidase A prepared by the method of Allan *et al.* (1964) was employed. The zinc content of all preparations was between 0.97 and 1.03 g-atoms/mole, based on a molecular weight of 34,600 (Bargetzi *et al.*, 1963) and a molar absorptivity of ϵ_{278} 6.42 × 10^4 M^{-1} cm^{-1} (Simpson *et al.*, 1963). The proteolytic coefficient of the native enzyme, C, was 35–40, while the esterase activity, k, was 7.2–8.2 × 10^3 min^{-1} when assayed as described below. ^{131}I was obtained as a carrier-free solution of NaI from Isoserve Corp., Cambridge, Mass. All chemicals were reagent grade and were used without further purification except for iodine which was resublimed, ICl which was redistilled, and acetylimidazole which was recrystallized from isopropenyl acetate. Precautions to prevent contamination with adventitious metal ions were taken as previously reported (Thiers, 1957).

Methods

Peptidase activity was determined using carbobenzoxyglycyl-L-phenylalanine (Mann Research Laboratories) as substrate (Coleman and Vallee, 1960). Activity is expressed as an apparent proteolytic coefficient C defined as log (a_0/a) per min per μmole of enzyme, where a_0 and a represent the concentration of substrate at time 0 and time t, respectively. The assays were performed at 0° in 0.02 sodium Veronal–1.0 M NaCl buffer, pH 7.5, except where otherwise indicated. C was calculated from the linear portion of the

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[1] Abbreviations used: CGP, carbobenzoxyglycyl-L-phenylalanine; HPLA, hippuryl *dl*-β-phenyllactate.

first-order plots observed when hydrolysis did not exceed 15%.

Esterase activity was determined as previously described (Simpson *et al.*, 1963). Assays were performed at 25° with 3 ml of 0.01 M hippuryl *dl*-β-phenyllactate in 0.2 M NaCl–0.005 M Tris hydrochloride at pH 7.5. Activities are expressed as zero-order velocity constants, k, with units of μequiv of H^+ released/min per μmole of enzyme.

Protein concentrations were determined by absorbance at 278 mμ. The molar absorptivities of modified carboxypeptidases were: acetylcarboxypeptidase, ϵ_{278} 5.75 × 10^4 M^{-1} cm^{-1} and iodocarboxypeptidase, ϵ_{278} 6.42 × 10^4 M^{-1} cm^{-1}. The protein weights employed to calculate these values were determined by precipitation of the enzyme with 10% trichloroacetic acid and drying to constant weight at 104° (Hoch and Vallee, 1953).

A Beckman DU spectrophotometer or a Zeiss PMQII spectrophotometer was used for measurements of absorbance at discrete wavelengths, while a Cary Model 15 MS automatic recording spectrophotometer was employed for determination of absorption spectra. A Radiometer pH meter (Model TTT 1) equipped with a general purpose glass electrode was employed to determine pH. Isotopes were measured by counting in a Tracerlab well-type scintillation detector.

Optical rotatory dispersion was measured at room temperature in 0.5-mm cells containing protein solutions of a concentration of 6.2 mg/ml, using a Cary Model 60 automatic recording spectropolarimeter. Electrophoresis was carried out at 2° in a Spinco Model H electrophoresis and diffusion apparatus using a protein concentration of 8 mg/ml in 0.1 M Tris–1 M NaCl at pH 8.5 with a field of 0.3 v/cm. Ultracentrifugation was carried out in a Spinco Model E analytic ultracentrifuge at 23° using a protein concentration of 7.9 mg/ml, in the same buffer as above.

Amino acid analyses were performed with a Spinco Model 120 B analyzer utilizing the chromatographic procedures of Spackman *et al.* (1958). Acid hydrolysis was carried out in sealed, evaculated tubes with constant boiling HCl at 105° for 24 hr; 3 μl of phenol was added to each tube to prevent halogenation of tyrosine. Alkaline hydrolysis was performed with barium hydroxide for 18 hr at 105° as described by Ray and Koshland (1962). Tryptophan was determined on unhydrolyzed protein by the method of Spies and Chambers (1949).

Spectral titrations of the native and iodinated proteins were carried out as described by Edelhoch (1962) or by dilution of the enzyme in 0.001 M Tris hydrochloride–2 M NaCl into buffers of pH values varying from 4 to 13 prepared with Tris, glycine, and sodium chloride. The change in molar absorptivity for tyrosine on ionization of the phenolic hydroxyl, $\Delta\epsilon_{295}$, was 2.1 × 10^3 M^{-1} cm^{-1}, identical with the value reported for polytyrosine (Katchalski and Sela, 1953).

Carboxypeptidase was iodinated by addition of a 6–30-fold molar excess of freshly prepared 5 × 10^{-2} M I_2 in 0.5 M KI to a solution of carboxypeptidase (6 mg/ml) in 0.02 M sodium Veronal–2 M NaCl, pH 7.5, and 4°. The reaction was allowed to continue for varying lengths of time and was terminated by addition of sufficient 0.1 M sodium thiosulfate to decolorize the solution. Iodination with ICl was performed by addition of varying molar excesses of reagent to a solution of 15 mg/ml of carboxypeptidase in 0.02 M Veronal–2 M NaCl buffer at 0° and pH 7.5.[2] Modification was virtually instantaneous. Excess reagent was destroyed after 2 min by addition of sodium thiosulfate. Carboxypeptidase was acetylated with a 60-fold molar excess of *N*-acetylimidazole for 30 min at 23° in 0.02 M sodium Veronal–1 M NaCl buffer, pH 7.5 (Simpson *et al.*, 1963). In some experiments, β-phenylpropionate was employed as a protective agent. Activities were determined after suitable dilution or, alternatively, the protein was dialyzed *vs.* either the Veronal buffer or 0.001 M Tris HCl–1 M NaCl buffer, pH 7.5.

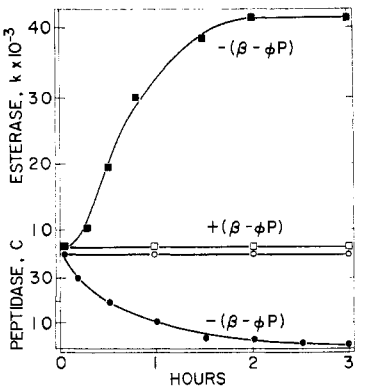

FIGURE 1: Progression of changes in esterase (■) and peptidase (●) activities during iodination of carboxypeptidase (6 mg/ml) with a 25-fold molar excess of I_3^- in the presence (□,○) and absence (■,●) of 0.02 M β-phenylpropionate, as indicated. Iodination was performed as under Experimental Section, and assays were performed following 24 hr of dialysis *vs.* three changes of 0.02 M Veronal–1 M NaCl, pH 7.5.

Results

Iodination with Iodine. Iodination of carboxypeptidase with a 25-fold molar excess of iodine at pH 7.5 and 4° markedly alters the catalytic specificity and hydrolytic rates of the enzyme. Esterase activity, measured with HPLA as the substrate, rapidly increases to a value five to six times that observed for the native enzyme. Concomitantly, peptidase activity measured with CGP is nearly abolished. There are no further alterations of activities when the reaction is allowed to continue for up to 3 hr (Figure 1).

An increase from 25- to 30-fold molar excess of iodine neither increases the rate nor the magnitude of the functional changes ultimately achieved. When less

[2] The authors are indebted to Dr. M. Koshland for communication of this method prior to its publication.

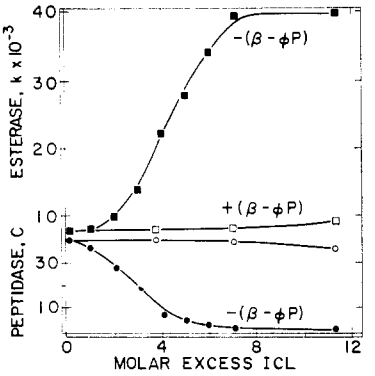

FIGURE 2: Effects of variation of the molar excess of iodine monochloride in the presence (□,○) and absence (■,●) of 0.1 M β-phenylpropionate on esterase (■) and peptidase (●) activities. Iodinations were performed at 0° in 0.02 M Veronal–2 M NaCl buffer, pH 7.5, for 5 min. Activities were determined as under Experimental Section after dilution.

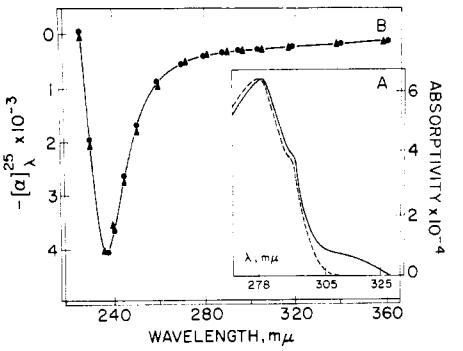

FIGURE 3: Ultraviolet absorption spectrum (A) of native (- - -) and iodinated (——) carboxypeptidase and optical rotatory dispersion (B) of native (●) and iodinated (▲) carboxypeptidase. The enzyme was iodinated with a 25-fold molar excess of I_3^- as under Experimental Section, and following dialysis, diluted in 0.01 M Tris–1 M NaCl, pH 7, to a concentration of 1.5×10^{-5} M for spectral examination.

than a 25-fold molar excess of iodine was used, maximal changes in esterase or peptidase activities did not occur during a 2-hr reaction period. Iodination in the presence of 0.02 M β-phenylpropionate, a competitive inhibitor of carboxypeptidase, failed to alter the activities of the native enzyme (Figure 1).

Carboxypeptidase was exposed to a 25-fold molar excess of iodine at pH 6 and 9, respectively, and the activities of the resultant iodoenzymes were then measured at pH 7.5. Iodination at pH 6 for 1 hr does not increase esterase activity, but peptidase activity decreases slightly. At pH 9, however, within 8 min after addition of iodine, esterase activity increases to 475% of the control value but progressively decreases thereafter. As judged by the changes in activities, the rate of iodination at pH 9 is greater than at pH 7.5, but the maximal enzymatic changes ultimately observed are virtually the same.

Modification with ICl. Iodination of carboxypeptidase with ICl incurs almost instantaneous functional consequences. The effects of iodination with differing molar excesses of ICl at pH 7.5 are shown in Figure 2. A sevenfold molar excess of ICl increases esterase activity to almost six times the control, while peptidase activity approaches zero. When such modifications are performed in the presence of 0.1 M β-phenylpropionate the activities of the native enzyme change only to a minor extent; molar excesses of ICl up to 10-fold increase esterase activity to about 120% while peptidase activity decreases to 90% of the control (Figure 2). When the molar excess of ICl is increased further, both activities decrease in spite of the presence of the protective agent.

Modification with ICl at pH 9 also increases esterase activity. However, a greater excess of reagent is required. A 14-fold molar excess of ICl, optimal at this pH, increases esterase activity to only 475% of the control.

Reversible Inactivation by Removal of Zinc. The removal and restoration of zinc reversibly inactivates iodocarboxypeptidase as has been reported for native and acetylcarboxypeptidase (Coombs *et al.*, 1964). Dialysis of iodocarboxypeptidase *vs.* 2×10^{-3} M, 1,10-phenanthroline at pH 7 removes zinc, and hence abolishes esterase activity. On addition of zinc equimolar with the iodoapoenzyme, the metal is bound, and the characteristic esterase activity of iodocarboxypeptidase is regained. The readdition of zinc to the iodinated enzymes does not restore peptidase activity.

Composition of Iodocarboxypeptidase. Amino acid analyses were performed in triplicate on native carboxypeptidase and on the enzyme iodinated in the presence and absence of β-phenylpropionate. Both alkaline and acid hydrolyses were performed. Tryptophan could not be determined in the alkaline hydrolysate due to the presence of iodotyrosine which emerges in an overlapping position with tryptophan in the standard analytic procedure employed (Spackman *et al.*, 1958). Therefore, tryptophan was determined by reaction with dimethylaminobenzaldehyde in sulfuric acid (Spies and Chambers, 1949), and iodotyrosine was estimated from the loss of tyrosine in alkaline hydrolysates and by spectrophotometric titration. Cysteine was not determined.

The results of the analyses are shown in Table I. As shown by the loss of tyrosine in alkaline hydrolysates, five to six tyrosyl residues were modified both in the presence or the absence of β-phenylpropionate. Methionine, serine, threonine, phenylalanine, and tryptophan were recovered fully and neither histidine nor tyrosine was lost on acid hydrolysis. Both Koshland *et al.* (1963) and Glazer and Sanger (1964) found that iodohistidine was not deiodinated significantly during acid hydrolysis. Based on these reports and the recovery of the eight histidines known to be present in

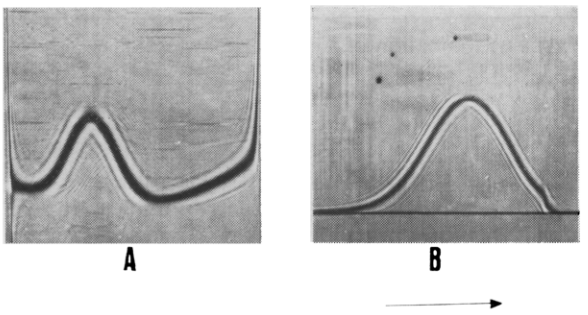

FIGURE 4: Sedimentation (A) and electrophoretic (B) patterns of iodocarboxypeptidase. (A) Sedimentation was from left to right. Photograph was taken 76 min after reaching rotor speed of 59,760 rpm. The sample was iodinated as in Figure 3A and centrifugation was carried out as under Experimental Section. (B) Pattern after 15 hr at 0.3 v/cm in 0.01 M Tris–1 M NaCl, pH 8.5. The enzyme was prepared as in Figure 3A. The anode is on the right and the direction of motion is as indicated.

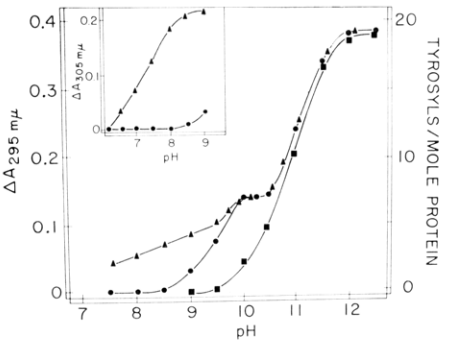

FIGURE 5: Spectral titration of native carboxypeptidase in aqueous buffer (●), and in 8 M urea (■), and iodinated carboxypeptidase (▲) in aqueous buffers. The enzyme was iodinated with an eightfold molar excess of ICl as under Experimental Section and following dialysis diluted to 1×10^{-5} M for the spectral titration. The inset shows the spectrotitration at 305 mμ of the iodinated (▲) and native (●) enzymes.

TABLE 1: Comparative Amino Acid Composition of Native and Iodinated Carboxypeptidase.[a]

Amino Acid	Native	Iodinated	
		Unprotected[b]	Protected[c]
Lys	15.1	14.9	14.7
His	8.1	8.0	7.7
Arg	10	10	10
Asp	27.2	26.7	27.0
Thr	22.8	22.5	22.9
Ser	28.1	28.0	28.4
Glu	25.1	24.8	25.0
Pro	10.4	10.9	11.1
Gly	22.8	23.7	22.6
Ala	19.0	18.4	19.5
Val	15.4	14.8	15.8
Met	3.0	3.0	3.1
Ileu	18.0	18.3	19.0
Leu	23.3	25.0	23.3
Phe	14.9	15.3	14.8
Tyr	18.4	18.6	18.5
Met[d]	3.0	2.8	2.9
Tyr[d]	19.4	13.1	13.6
Try[e]	7.9	8.0	8.1

[a] Results are expressed as moles of amino acid/mole of enzyme and are calculated on the basis of 10 arginines/mole. [b] Iodinated with a 20-fold molar excess of I_3^-, pH 7.5, 4°, 2.5 hr. [c] Iodinated in the presence of 0.02 M β-phenylpropionate. [d] Alkaline hydrolysate. [e] Colorimetric determination with *p*-dimethylaminobenzaldehyde.

carboxypeptidase, these data do not permit the conclusion that significant iodination of histidyl residues has occurred. Thus, tyrosine seems to be the only residue which has been modified under the conditions employed.

As measured by ^{131}I incorporation 4.8–6.2 equiv of iodine is incorporated/mole of protein during modification, values which correspond closely to between five and six tyrosyl residues iodinated, as shown by loss of tyrosine in the alkaline hydrolysates. This correlation suggests that only minimal formation of diiodotyrosine has occurred. Only trace amounts of diiodotyrosine were, in fact, detected upon two-dimensional paper chromatography of alkaline hydrolysates of the modified proteins (1-butanol–acetic acid–water, 120:30:50, followed by 95% ethanol–ammonia, 19:1). These analytical findings are in accord, moreover, with those obtained on spectrophotometric titration (*vide infra*).

Physicochemical Characterization of Iodocarboxypeptidase. Iodination alters the ultraviolet absorption spectrum of carboxypeptidase in a manner consistent with iodination of tyrosyl residues (Figure 3A). The absorption maximum of the protein shifts to longer wavelengths and the absorption between 290 and 330 mμ is increased, due to the formation of iodotyrosyl residues. In contrast to these changes in the absorption spectrum, no significant alterations in optical rotatory dispersion were observed on iodination of carboxypeptidase. Within the limit of experimental error the dispersion of the native and iodoenzymes are identical, with the trough of the negative intrinsic Cotton effect at 235.5 mμ and the crossover to positive rotation at 224.5 mμ (Figure 3B).

Similarly, ultracentrifugation of iodocarboxypeptidase failed to demonstrate heterogeneity or differences from the native enzyme. The modified protein sedimented as a single symmetric peak with an approximate sedimentation constant of 3.4 S (Figure 4A). Moving

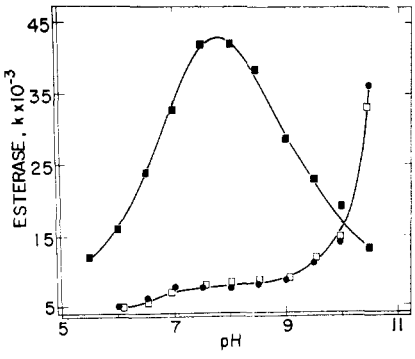

FIGURE 6: Esterase pH–rate profiles for native (●) and iodinated (■) carboxypeptidases, and iodinated carboxypeptidase prepared in the presence of 0.02 M β-phenylpropionate (□). The enzyme was iodinated with a 25-fold molar excess of I_3^- as under Experimental Section. Activities were measured using HPLA for substrate, as described under Experimental Section, at the pH on the abscissa. Assays were not performed at pH values >10.5 due to rapid loss of zinc from the enzyme.

TABLE II: Iodination of Acetylcarboxypeptidase.

Sample[a]	Activity: Peptidase (C)	Activity: Esterase ($k \times 10^{-3}$)
(1) Native	40	7.5
(2) Acetyl[b]	2	44.0
(3) Iodoacetyl[c]	1	43.0
(4) Deacetylated iodoacetyl[d]	39	7.6

[a] Modifications performed consecutively on a single sample of enzyme. [b] Native carboxypeptidase was acetylated with a 60-fold molar excess of *N*-acetylimidazole as under Experimental Section. [c] Sample 2 was iodinated with a 25-fold molar excess of iodine for 2.5 hr. [d] Sample 3 was treated with 1 M hydroxylamine for 10 min, pH 7.5, 23°.

boundary electrophoresis at pH 8.5 showed the presence of about 4% of a material which moved more rapidly toward the anode than the major protein boundary (Figure 4B).

The spectral titration of native carboxypeptidase demonstrates the presence of seven to eight tyrosyl residues which ionize with a pK_a of 9.5, and 11–12 residues which ionize irreversibly at pH values >10.5 (Figure 5) (Simpson and Vallee, 1963). The tyrosyl residues titrating with a pK_a of 9.5 may be classified as free and are presumably at the surface of the molecule, while those which titrate at the higher pK_a are buried and are presumably located in the interior of the folded protein. The presence of 8 M urea normalizes the spectral titration. The spectral titration of iodocarboxypeptidase is unlike that of thyroglobulin (Edelhoch, 1962) in that ionization of iodotyrosyl residues appears to change the absorbance at 295 mμ, as evidenced by the increased absorbance at this wavelength at pH values <9. Nonetheless, two free residues are titrated in the iodinated enzyme with a p*K* like those in the native enzyme and all the buried tyrosyls are titrated as in native carboxypeptidase. Spectral titration at 305 mμ indicates the presence of approximately four monoiodotyrosyl residues/mole of enzyme (Figure 5 inset), using $\Delta\epsilon_{305} = 4100$ for ionization of this residue (Edelhoch, 1962). Similar spectral titration from pH 6 to 8 at 325 mμ demonstrates the presence of approximately one diiodotyrosyl residue/mole of protein.

Iodination of Acetylated Carboxypeptidase. The lack of effect of iodination on the activities of acetylcarboxypeptidase suggests that both procedures modify the same functional residues of the enzyme. Acetylation of carboxypeptidase with a 60-fold excess of acetylimidazole (Simpson *et al.*, 1963) increases esterase activity to 600% and decreases peptidase activity to <5% of control values (Table II). Iodination of this acetylcarboxypeptidase does not significantly alter these activities. Deacetylation with hydroxylamine of the protein exposed to this double modification restores activities to those of the native enzyme. Thus, prior acetylation completely prevents the functional effects of iodination. Therefore, two separate lines of evidence indicate that iodination of a tyrosyl residue(s) at the active center of carboxypeptidase leads to the observed activity changes. But while acetylation has been shown to selectively alter two tyrosyl residues at the active center, analogous attempts to ascertain the number of tyrosyl residues whose iodination provokes these activity changes, have been unsuccessful. Protection of the active center from modification with stable and isotopic ICl and iodine by inhibitors and substrates failed to reveal differences, perhaps due to exchange and migration of iodine (Glazer and Sanger, 1964).

Spectral titrations of carboxypeptidase iodinated in the presence and the absence of β-phenylpropionate have thus far failed to demonstrate any significant difference in the number of residues modified, although the enzymatic activities of the two iodinated proteins differ markedly (*vide supra*). Further, investigation of the amount of radioactive iodine incorporated into the protein consequent to modification have demonstrated a difference of only 0.6 (or less) equiv of iodine/mole of protein between protected and nonprotected samples.

Kinetic Properties of Iodocarboxypeptidase. The esterase activity profile of native carboxypeptidase increases continuously from pH 6 to 9.5 and then more sharply up to pH 10.5 (Riordan and Vallee, 1963). Beyond this the activity falls off rapidly but zinc is lost simultaneously. The activity profile of iodocarboxypeptidase is bell shaped with a maximum at pH 7.75 (Figure 6), and points of inflection at pH 6.8 and 9. The pH–rate profile of the enzyme modified in the

presence of β-phenylpropionate, however, is indistinguishable from that of the native enzyme.

The esterase activity of native carboxypeptidase decreases as the concentration of *dl*-HPLA increases from 0.001 to 0.1 M (Snoke *et al.*, 1948; Snoke and Neurath, 1949; McClure *et al.*, 1964; Bender *et al.*, 1965), a phenomenon attributed to substrate inhibition. At substrate concentrations of <0.001 M, Michaelis–Menten kinetics are obeyed, with kinetic constants of K_m(app) = 5.1×10^{-5} M and K_0 = 2.8×10^4 min^{-1} (McClure *et al.*, 1964) or K_m(app) = 8.8×10^{-5} M and k_{cat} = 3.47×10^4 min^{-1} (Bender *et al.*, 1965). Preliminary kinetic investigations of iodocarboxypeptidase over the substrate range from 0.002 to 0.08 M *dl*-HPLA indicate that like acetylation (Riordan *et al.*, 1963; Bender *et al.*, 1965) iodination relieves substrate inhibition of the native enzyme at 0.01 M HPLA, the conventional (Snoke and Neurath, 1949) assay concentration. The maximal observed velocity for ester hydrolysis by the iodoenzyme is 4.8×10^4 min^{-1} at 0.02 M substrate, a value significantly higher than either of the calculated maximal velocities reported. Detailed kinetic studies of various metalloiodocarboxypeptidases are in progress.

Discussion

Iodination alters the catalytic activity of carboxypeptidase in a manner similar to that observed on acylation. Under the conditions of assay the esterase activity is not only maintained, but actally increases to a value five to six times greater than that of the native enzyme. Concomitantly, peptidase activity is virtually lost. The presence of β-phenylpropionate during the modification reaction prevents these functional changes (Figures 1 and 2). Equilibrium dialysis with ^{14}C-labeled β-phenylpropionate (Coleman and Vallee, 1964) shows that it does not bind to the apoenzyme. Analogous results have been obtained with phenyl acetate (Rupley and Neurath, 1960). Since β-phenylpropionate prevents the acetylation of tyrosyl residues critical for enzymatic activity (Simpson *et al.*, 1963; Riordan and Vallee, 1963), it would appear that these are located within the limited area which this agent covers when bound at the active site.

Both ultracentrifugation and electrophoresis demonstrate that the modified protein is >95% homogeneous. Further, the conformation of the modified and native enzymes are indistinguishable within the limits demonstrable by optical rotatory dispersion. Thus, if the changes in enzymatic activities are accompanied by changes in conformation, such alterations are not readily detected by these means. Such physicochemical studies are considered prerequisite for the interpretation of the kinetics of a chemically modified enzyme, the assignment of chemical changes to specific moieties of the active center, and the possible role of conformational changes in the alterations of enzymatic activities.

The lack of specificity of iodine as a reagent for modification of proteins has been discussed (Frankel-Conrat, 1959). Iodine oxidizes cysteinyl, tryptophanyl, seryl, threonyl, and methionyl residues and halogenates histidyl and tyrosyl residues, delineating the possibilities to be considered if the results here obtained are to be interpreted in terms of the modification of primary structure.

The specificity of iodine monochloride for tyrosyl substitution may be greater than that of iodine, since 15–16 tyrosyl residues of rabbit γ-globulin are iodinated before other residues are modified (Koshland *et al.*, 1963), though ICl has been found to iodinate both tyrosine and histidine at pH 8 (Glazer and Sanger, 1964). Iodine monochloride, like iodine, a strong oxidizing agent, might oxidize yet other amino acid residues.

The sole cysteinyl residue of carboxypeptidase to which zinc is bound apparently is not oxidized, since its destruction would result in the loss of the metal and thereby abolish all enzymatic activity of the enzyme. Iodoapocarboxypeptidase, like apocarboxypeptidase, is catalytically inactive, hence the cysteinyl residue which binds zinc is an essential component of the active center both of the native and of the chemically modified enzyme. Studies of the interaction of different metals with iodocarboxypeptidase (Vallee, 1964) show that both the particular metal and the nature of the organic groups in the active center determine the functional properties of the enzyme. Thus, mercury carboxypeptidase hydrolyzes esters but not peptides. Mercuriiodocarboxypeptidase, however, is not catalytically active toward either one (Vallee, 1964; Coleman *et al.*, 1964).

The precise chemical nature of the atypical sulfur residue of the enzyme is unknown, hence its iodination cannot be ruled out *a priori*. However, alkylation of this residue after reduction with sodium borohydride does not alter activity nor does β-phenylpropionate prevent its reduction (Walsh *et al.*, 1962).

Although iodination can oxidize methionine and aliphatic hydroxy-containing amino acids, a high I^-/I_2 ratio at acidic pH values is required to achieve optimal conditions for oxidation, circumstances quite dissimilar to those which induce functional changes of carboxypeptidase. Further, amino acid analyses show no change in the content of methionine, serine, or threonine (Table I).

Recently, oxidation of a single tryptophanyl residue has been demonstrated to be the cause of loss of activity of lysozyme upon iodination (Hartdegen and Rupley, 1964). Comparison of analyses of native and iodinated carboxypeptidase do not show any losses of tryptophan under the conditions of iodination employed (Table I), thus eliminating oxidation of the indole ring as a basis of the enzymic consequences of iodination.

Aside from tyrosine, histidyl residues are most likely to be modified by iodination. Histidine is iodinated at alkaline pH, although under similar conditions the rate of modification is slower than that observed for tyrosine (Glazer and Sanger, 1964). Nonetheless, histidine is modified during iodination of pepsin-inactivated ribonuclease. Although the product of modification was not identified, histidine was lost in acid hydrolysates (Fujioka and Scheraga, 1965). The

stability of iodohistidine to acid hydrolysis has been noted previously (Glazer and Sanger, 1964). In the present study, no histidine was lost on amino acid analyses of the iodinated protein. These data would seem to center attention on tyrosine in searching for the chemical basis of the enzymatic changes induced by iodination of carboxypeptidase. This view is consistent, of course, with the results of previous chemical modification.

Although it seems certain that modification of tyrosyl residues by iodination provokes the activity changes similar to those on acetylation of carboxypeptidase (Simpson *et al.*, 1963; Riordan and Vallee, 1963), attempts to define precisely the stoichiometry of the modification reaction have been unsuccessful so far. During acetylation of native carboxypeptidase with acetylimidazole or acid anhydrides, two reactive tyrosyl residues at the active center are selectively protected by the presence of substrate or competitive inhibitors (Simpson *et al.*, 1963; Riordan and Vallee, 1963). Similarly, the active center tyrosyl residues are clearly more labile to deacylation with hydroxylamine than are the remainder of the acylated surface tyrosyl residues of the acetylcarboxypeptidase (Simpson *et al.*, 1963). During iodination, β-phenylpropionate completely prevents the *functional* changes consequent to modification. However, by spectral titration, amino acid analysis, and incorporation of ^{131}I the number of residues modified in the absence of β-phenylpropionate is only about 0.6/mole greater than that modified in its presence. In these experiments, utilizing a limiting concentration of modifying agent, the reactivity toward iodine of the active center tyrosyl residues and of the remainder of the surface tyrosyl residues of carboxypeptidase must be quite similar, in contrast to their differing reactivity to the milder acylating agents.

The stoichiometry of iodine incorporation in the presence and absence of β-phenylpropionate observed in a study of peptides isolated from carboxypeptidase iodinated both with ^{125}I and ^{131}I was quite similar (Roholt *et al.*, 1965). In samples modified under the mildest conditions employed in that study, 13.03 equiv of I/mole of enzyme was incorporated in the presence of the inhibitor and 13.32 in its absence, a difference of 0.3, even less than reported here, though of similar magnitude. Differential labeling of peptides with ^{125}I and ^{131}I between the protected and unprotected samples was thought to be consistent with modification of two "active center tyrosyl residues" in the unprotected sample (Roholt *et al.*, 1965), in accord with our previous studies by acetylation (Simpson *et al.*, 1963). Under the conditions which were employed for iodination, *viz.*, a 20-fold molar excess of ICl, pH 9.0, some destruction of the conformation of the native molecule must have occurred, since at least eleven tyrosyl residues were iodinated, although only seven are free in native carboxypeptidase. Further, under these conditions, 0.1 M β-phenylpropionate no longer provides full protection against the functional consequences of iodination. Neither the results of Roholt *et al.* (1965) nor our present data add decisive information on the number of tyrosyl residues at the active site; and the interpretation that two tyrosyl residues are involved in the activity of carboxypeptidase must remain dependent largely on the results of acylation (Simpson *et al.*, 1963; Riordan and Vallee, 1963, 1964).

The loss of peptidase activity upon iodination of carboxypeptidase reflects, at least in part, decreased substrate binding, since it has been shown that, in contrast to the native enzyme, apoiodocarboxypeptidase does not form complexes with peptide substrates to prevent reformation of the holoenzyme on addition of zinc (Vallee, 1964). The increased esterase activity of iodocarboxypeptidase observed under the conventional conditions of assay, *i.e.*, 0.01 M *dl*-HPLA, derives at least in part from a loss of substrate inhibition. However, the V_{max} of iodocarboxypeptidase, 4.8×10^4 min^{-1}, *vs.* 2.8×10^4 min^{-1} (McClure *et al.*, 1964) for the native enzyme, is greater than can be accounted for by the removal of substrate inhibition.

The striking differences in the pH dependence of esterase activity of native and iodocarboxypeptidase further emphasize the alterations induced by iodination. The sharp increase in activity of the native enzyme at pH values above 9.5 may either be due to increased hydroxide ion concentration, as previously suggested (Riordan and Vallee, 1963), or may be related to an ionization of the enzyme, perhaps a tyrosyl residue. But the esterase activity of iodocarboxypeptidase is not proportional to hydroxide ion concentration. Further, the changes in activity of the iodoenzyme as a function of pH cannot be attributed to irreversible physicochemical changes since preincubation of the enzyme at discrete pH values from 6 to 9.5 for 1 hr does not alter the activity assayed at pH 7.5. The observed changes in pH dependence may reflect the altered pK of phenol ionization induced by iodination. A difference in the rate-limiting step of ester hydrolysis in the native and modified enzymes cannot be excluded as the basis for this phenomenon. These and other alternatives are presently under investigation.

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