Azocarboxypeptidase: Functional Consequences of Tyrosyl and Histidyl Modification*

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ABSTRACT: At pH 8.8 5-diazo-1H-tetrazole curtails the dual specificity of carboxypeptidase A. On addition of up to eightfold molar excess of reagent, esterase activity increases to approximately 200%, while peptidase activity is maintained at about 90% of the control. As the molar ratio of the reagent to enzyme increases, esterase activity is not altered further, but peptidase activity progressively decreases to become less than 1% when the ratio of reagent to enzyme is 45. In the presence of the competitive inhibitor β-phenylpropionate, the changes in activities are prevented, suggesting that the coupling reaction involves residues at the active center of the enzyme. The dual specificity of carboxypeptidase serves as an important index for the definition of optimal conditions of reaction. Low concentrations of reagent, as here employed, considerably narrow the choice among the amino acid residues which might be responsible for enzymatic changes; however, the spectral changes are not sufficiently specific for the decisive identification of the amino acid residue(s) involved. However, specificity can be increased by combining coupling with other modifications of known chemical, spectral, and enzymatic consequences, *e.g.*, acetylation or iodination. Simultaneously amino acid analysis permits further discrimination. Previous and present evidence jointly suggest that modification of tyrosine and histidine causes the observed changes in activity.

Numerous studies indicate that histidyl residues participate in the mechanism of action of many enzymes. The effect of pH on enzymatic rates and the consequences of chemical modification on activity constitute the principal support for such conclusions. The paucity of suitable reagents, specific for the imidazole group of enzymes, has handicapped such studies. Investigations of the role of histidine in the mode of action of the hydrolytic enzymes of the pancreas have generated a great deal of the current knowledge in regard to the participation of this residue in the mechanism of enzyme action (Barnard and Stein, 1958), though for carboxypeptidase A there has been very little definitive information. The pH–rate profile of peptide hydrolysis by carboxypeptidase A is bell shaped with an inflection point at pH 6.7, close to the pK of histidine. This and other considerations have been employed to implicate histidyl residues in the catalytic mechanism of this enzyme (Neurath and Schwert, 1950; Vallee *et al.*, 1963). However, alkylating agents successfully employed for this purpose, *e.g.*, on ribonuclease (Barnard and Stein, 1959; Gundlach *et al.*, 1959), have failed so far to induce discernible chemical alterations or to change the activity of carboxypeptidase irreversibly (Coombs *et al.*, 1964).

Histidyl residues of proteins have been coupled with a number of diazonium compounds, but lack of specificity has largely rendered such reagents of limited value for purposes of definitive identification. One such compound, diazonium-1H-tetrazole (DHT),[1] has been described recently (Horinishi *et al.*, 1964), and in a previous paper we have detailed the spectral properties of some of its derivatives (Sokolovsky and Vallee, 1966a). The present investigation was undertaken to examine the reaction of DHT with carboxypeptidase, and to define optimal conditions for the exploration of the active center of this enzyme. For such investigations the molar excess of DHT is critical and must be much lower than that required for the total analysis of tyrosine and histidine in proteins (Sokolovsky and Vallee, 1966a).

Coupling of carboxypeptidase with a 30-fold molar excess of DHT alters both esterase and peptidase activities. Acetylation of carboxypeptidase also changes these two activities owing to the modification of two tyrosyl residues, but the enzymatic characteristics of the two modified enzymes are quite different. The present evidence suggests that, in addition to tyrosine, another residue, perhaps histidine, is modified by DHT and may be involved in the mechanism of action

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[1] Abbreviations used: DHT, 5-diazonium-1H-tetrazole; HPLA, hippuryl-DL-β-phenyllactate; [(CPD)Zn], carboxypeptidase; [(AcCPD)Zn], acetylcarboxypeptidase; [(AzoCPD)Zn], azocarboxypeptidase; [Azo(AcCPD)Zn], acetylcarboxypeptidase which is then coupled with DHT.

of carboxypeptidase. A preliminary report has been given (Sokolovsky and Vallee, 1966b).

Materials

Carboxypeptidase A, prepared by the method of Anson (1937) and obtained from Worthington Biochemical Corp., had a zinc-to-protein ratio between 0.98 and 1.03 g-atoms/mole based on a molecular weight of 34,600 (Bargetzi *et al.*, 1963). *N*-Acetylimidazole was obtained from K and K Laboratories, 5-amino-1H-tetrazole from Aldrich Chemical Corp., and iodine monochloride was a gift from Dr. W. Cobain. Precautions to prevent contamination by adventitious metal ions were taken throughout these studies (Thiers, 1957).

Methods

Peptidase activity was determined using the synthetic substrate carbobenzoxyglycyl-L-phenylalanine (Mann Research Laboratories) (Elkins-Kaufman and Neurath, 1949; Coleman and Vallee, 1960), and is expressed as an apparent proteolytic coefficient, C, defined as $\log a_0/a$ per minute per micromole of enzyme, where a_0 and a represent the concentration of substrate at times 0 and t, respectively (Simpson *et al.*, 1963). The assays were performed at 0° in 0.02 M sodium Veronal–1.0 M NaCl buffer, pH 7.5, and C was calculated from the linear portion of the first-order reaction plots before hydrolysis exceeded 15%.

Esterase activity was determined by pH titration (Snoke *et al.*, 1948) with 0.1 M NaOH of the hydrogen ions released on hydrolysis using a pH-Stat (Radiometer, Copenhagen) and recorder (Ole Dich, Copenhagen). 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 buffer, pH 7.5. Activities are expressed as zero-order velocity constants, k, with units of equivalents of H^+ released per minute per mole of enzyme.

Protein concentration was measured by the absorbance at 278 mμ. The molar absorptivity of native carboxypeptidase A is 6.42×10^4 M^{-1} cm^{-1} (Simpson *et al.*, 1963).

Absorption Spectra. A Zeiss PMQ II spectrophotometer was used for absorbance measurements at single wavelengths, and spectra were obtained with a Cary Model 15 MS automatic recording instrument.

pH was measured with a Radiometer pH meter (Model pH M4) equipped with a Radiometer GK 2021 electrode.

Amino acid analyses were carried out with a Spinco Model 120 B automatic amino acid analyzer by the technique of Spackman *et al.* (1958). Acid hydrolysis was carried out in sealed, evacuated tubes with 6 N HCl at 105° for 24 hr; 5 μl of phenol was added to each tube to prevent halogenation of tyrosine. Tryptophan was determined on unhydrolyzed protein by the method of Spies and Chambers (1949).

DHT was prepared by diazotization of 1 g of 5-amino-1H-tetrazole dissolved in 23 ml of 1.6 M HCl with 0.7 g of sodium nitrite in 10 ml of water at 0° (Sokolovsky and Vallee, 1966a). Prior to the coupling reaction, the solution was adjusted to pH 5 with alkali. Concentrations were determined as described (Sokolovsky and Vallee, 1966a).

Coupling Reaction. A solution of DHT (1 ml) at the appropriate concentration was mixed with 9 ml of carboxypeptidase ($5–6 \times 10^{-5}$ M) dissolved in 0.67 M $KHCO_3$–1 M NaCl at pH 8.8. The time course of the effect of DHT on the activity was followed by removing aliquots from the reaction mixture, diluting 50- to 100-fold with 0.1 M Tris–1 M NaCl, pH 7.5, and assaying for peptidase and esterase activity. This procedure arrests further modification of the enzyme, since reaction with Tris effectively removes excess DHT. The spectrum of the reaction mixture was recorded directly. When necessary, bicarbonate ions and excess reagent were removed by dialysis against three changes of metal-free water for 18 hr at 4°. Azocarboxypeptidase is soluble in water in the absence of salt.

Acetylation with acetylimidazole was performed at room temperature by adding the reagent dissolved in 0.02 M sodiume Veronal–1 M NaCl (pH 7.5) to enzyme (2×10^{-5} M) dissolved in the same buffer (Simpson *et al.*, 1963).

Succinylation was carried out at 0–4° by adding a 50-fold molar excess of solid succinic anhydride to a well-stirred solution of carboxypeptidase (10 mg/ml) in 0.02 M sodium Veronal–1 M NaCl, pH 7.5, on the pH-Stat. During dialysis for 48 hr against the same buffer and pH, spontaneous desuccinylation of tyrosyl residues takes place as described (Riordan and Vallee, 1964).

Iodination was performed at 0° by adding an eightfold molar excess of iodine monochloride in 0.05 M Tris–1 M NaCl, pH 7.5, to a solution of the enzyme in the same buffer. The reaction was considered complete after 5 min (Simpson and Vallee, 1966).

Determination of Free Amino Groups. The degree of acylation of the free amino groups of carboxypeptidase was determined by means of the ninhydrin reaction using phenylalanine as a standard (Moore and Stein, 1948).

Metal Analysis. Zinc was determined by atomic absorption spectroscopy (Fuwa and Vallee, 1963).

Results

The spectral properties of mono- and bisazo derivatives of tyrosyl and histidyl residues have been evaluated recently (Sokolovsky and Vallee, 1966a), and the spectra of mono- and bisazo-*N*-acetyltyrosine and of bisazo-*N*-acetylhistidine are shown for reference in Figure 1.[2] Mono- and bisazo-*N*-acetyltyrosine as well as bisazohistidine contribute significantly to

[2] Monoazo and bisazo refer to the 5-azo-1H-tetrazole derivatives of histidyl and tyrosyl residues.

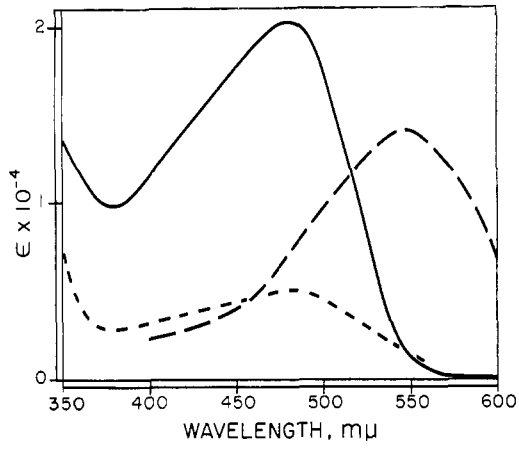

FIGURE 1: Absorption spectra of monoazo-*N*-acetyltyrosine (– – – –), bisazo-*N*-acetyltyrosine (— —), and bisazo-*N*-acetylhistidine (———), bicarbonate buffer, pH 8.8. The monoazotyrosyl derivative was obtained by treating 1×10^{-4} M *N*-acetyltyrosine with 1.2×10^{-4} M DHT, the bisazotyrosyl and bisazohistidyl with 3×10^{-2} M DHT, for 30 min at room temperature.

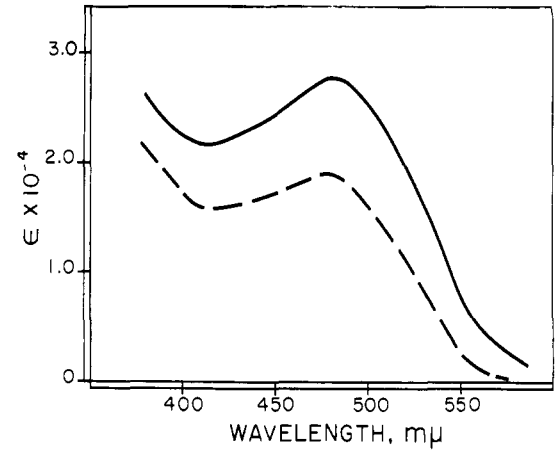

FIGURE 3: Absorption spectra of azocarboxypeptidase (———) and azoacetylcarboxypeptidase (— —), pH 8.8, 0.67 M $KHCO_3$–1 M NaCl, 0°. The native and acetylated enzymes were treated with a 30-fold molar excess of DHT, for 30 min, pH 8.8, 0°.

the absorption at 480 mμ. However, with a 30-fold molar excess of reagent over enzyme, the conditions generally employed here, only monoazotyrosine and bisazohistidine are apt to form (Sokolovsky and Vallee, 1966a).

In order to establish the composition of a mixture containing these two derivatives their contributions to the absorption at 480 mμ must be differentiated. This can be accomplished by acetylation of the tyrosyl derivative. On acetylation of monoazo-*N*-carbobenzoxytyrosine (Sokolovsky and Vallee, 1966a) with *N*-acetylimidazole the absorbance of the resultant *O*-acetyl derivative at 480 mμ decreases to approximately one-fourth[3] that of the unmodified material, ϵ_{480} 1300 (Figure 2). Since acetylation does not affect the spectrum of bisazo-*N*-acetylhistidine, *O*-acetylation of tyrosine can resolve the relative contributions of monoazotyrosyl and bisazohistidyl residues to the absorption at 480 mμ.

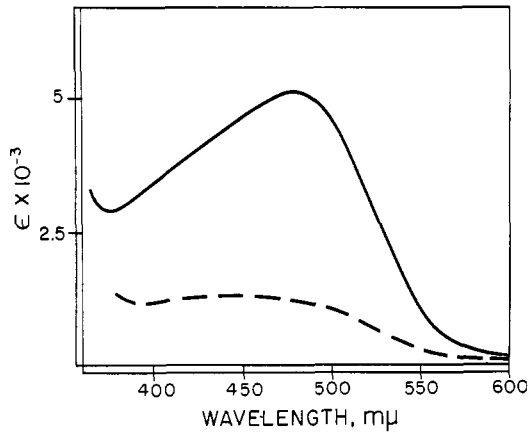

FIGURE 2: Absorption spectra at pH 8.8 of monoazo-*N*-carbobenzoxytyrosine before (———) and after (— —) reaction with *N*-acetylimidazole. Acetylation was performed with a 200-fold molar excess of reagent at pH 7.5, 0°; the pH of aliquots withdrawn was adjusted to pH 8.8 with 0.67 M $KHCO_3$ buffer.

Spectral Properties of Azocarboxypeptidase. Within seconds after coupling of a 30-fold molar excess of DHT with carboxypeptidase, a spectrum is generated with a maximum at 480 mμ (Figure 3); at 0° the reaction is complete within 30 min. The derivative is dialyzed for 24 hr against 0.02 M Veronal–1 M NaCl, pH 7.5, at 4°, and then acetylated with a 200-fold molar excess of *N*-acetylimidazole at 0° for 30 min. At pH 8.8 the molar absorptivity of the azoenzyme, ϵ_{480} 27,000, decreases to ϵ_{480} 21,500 for acetylazocarboxypeptidase.[3] Calculated on the basis of their known molar absorptivities, 1.8 monoazotyrosyl and 0.92 histidyl residues contribute to the absorbance of azocarboxypeptidase at 480 mμ.

Enzymatic Effects of Coupling. Coupling of carboxypeptidase with DHT at pH 8.8 markedly alters the activities of the enzyme. With an eight- to ninefold molar excess of reagent esterase activity increases to approximately 200% while the peptidase activity remains at about 90% of the control (Figure 4). As the molar ratio of DHT to enzyme increases, esterase activity is not altered further, but peptidase activity progressively decreases, to become less than

[3] On raising the pH from 7.5 to 8.8, the optimum for spectral measurements of the azo derivatives, the A_{480} of the resultant *O*-acetylmonoazotyrosine increases quite rapidly, probably owing to the hydrolysis of the active ester bond. Therefore, this rate is measured and extrapolated to zero time.

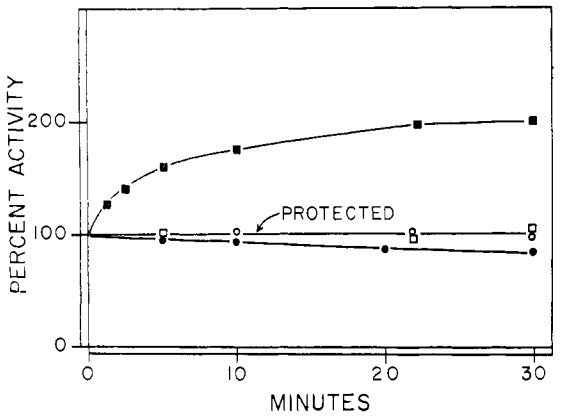

FIGURE 4: Progression of changes in esterase (■—■) and peptidase (●—●) activity during coupling of carboxypeptidase (5×10^{-5} M) with an eightfold molar excess of DHT, pH 8.8, in the presence (□—□, ○—○) and absence of 0.1 M β-phenylpropionate. Assays were performed as described under Methods.

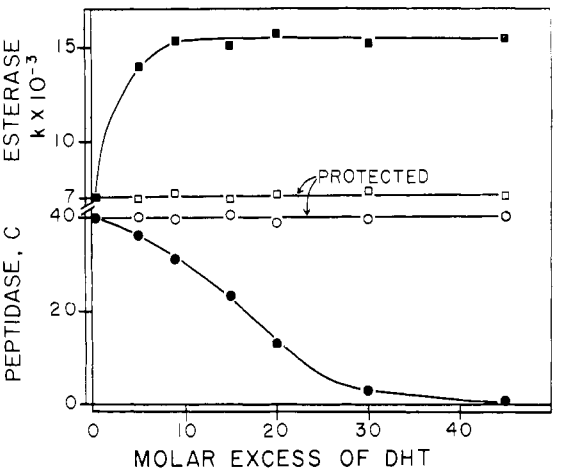

FIGURE 5: Effects of variation of the molar excess of DHT in the presence (□—□, ○—○) and absence of 0.1 M β-phenylpropionate on esterase (■—■) and peptidase (●—●) activities. Carboxypeptidase (5×10^{-5} M) was coupled with DHT at 0° in 0.67 M $KHCO_3$–1 M NaCl buffer, pH 8.8, for 30 min. Activities were determined as described under Methods.

1% when the molar ratio of DHT to enzyme is 45 (Figure 5). The enzyme remains stable for 24 hr when modified with a 30-fold molar excess for 30 min at 0° Hence, these conditions were adopted to standardize the procedure.

Dialysis of azocarboxypeptidase for 3 hr reduces its esterase activity to about 140% of the control, but does not alter peptidase activity. Coupling of carboxypeptidase with DHT in the presence of 0.1 M β-phenylpropionate prevents both the changes of catalytic specificity and those of the hydrolytic rates (Figure 5). This inhibitor apparently prevents coupling to the active-center residues of the enzyme, an observation analogous to that on acetylation (Simpson *et al.*, 1963; Riordan and Vallee, 1963) and iodination (Simpson and Vallee, 1966). The activity changes are not related to the metal content of carboxypeptidase. Azocarboxypeptidase, dialyzed exhaustively against metal-free distilled water at 4°, contains 1 g-atom of zinc, unaltered from and identical with the native enzyme.

The changes in esterase and peptidase activities of the enzyme coupled with a 30-fold molar excess of DHT at pH 8.8, 0° were correlated with the total absorption at 480 mμ (Figure 6). During the initial rise in absorption, esterase activity increases markedly and becomes constant thereafter, but the decrease of peptidase activity occurs over a much wider range of changes in A_{480}. The first phase corresponds either to modification of 1.1 monoazotyrosyl residue or, alternatively, of 0.3 bisazohistidyl residue based on an ϵ_{480} of 5100 and 20,500, respectively. The subsequent decrease in peptidase activity coincides with an increase in molar absorptivity of azocarboxypeptidase at 480 mμ of 20,000, corresponding either to the coupling of approximately one histidyl or, alternatively, of four tyrosyl residues.

Amino Acid Composition. In addition to tyrosine and histidine, DHT may also modify lysine, arginine, serine, and threonine, though the latter derivatives are not colored. Acid hydrolysis does not regenerate the free amino acids from the monoazo and bisazo derivatives of *N*-acetyltyrosine and of *N*-acetylhistidine (Sokolovsky and Vallee, 1966a).

A mixture of hippuryllysine, hippurylarginine, *N*-acetylserine, and *N*-acetylthreonine was treated with a 50-fold molar excess of DHT for 1 hr, pH 8.8. The mixture was subjected to acid hydrolysis and chromatographed on the Spinco amino acid analyzer. DHT

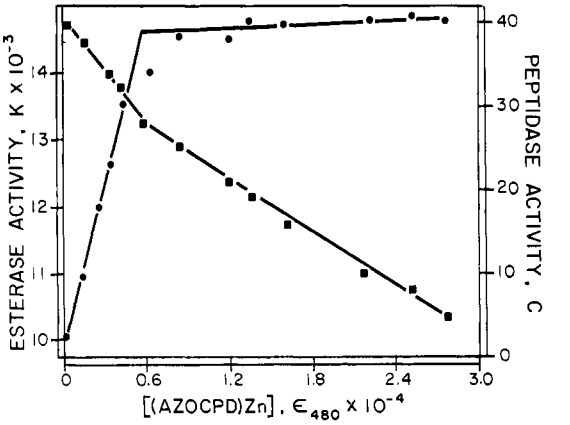

FIGURE 6: Correlation of the molar absorptivity ϵ_{480} with esterase (●—●) and peptidase (■—■) activities of carboxypeptidase coupled with a 30-fold molar excess of DHT for 30 min at pH 8.8, 0°.

itself does not yield ninhydrin-positive products other than ammonia. The product of DHT and hippurylysine quantitatively generates glycine but not lysine. In addition, two new ninhydrin-positive peaks emerge, one before glycine and the other before tyrosine. The modification of lysine, histidine, and tyrosine by DHT can be detected by virtue of their destruction. The product of DHT and hippurylarginine generates both arginine and glycine, but the amount of arginine is 89% of that of glycine, and no new ninhydrin peaks are found. Similarly, when corrected for destruction during hydrolysis, serine and threonine are recovered and no additional ninhydrin peaks are observed.

Amino acid analyses were performed on native carboxypeptidase and on enzyme coupled with 4-, 8-, 12-, 20-, and 30-fold molar excesses of DHT, both in the presence and absence of β-phenylpropionate. The analyses of the native enzyme and of that coupled with 8- and 30-fold molar excess of the reagent are shown in Table I. Save for tyrosine, histidine, and lysine, the amino acid composition of either azocarboxypeptidase does not differ significantly from that of the native enzyme. β-Phenylpropionate protects one tyrosyl residue from coupling with an eightfold molar excess of DHT, and additionally protects 0.9 histidyl residue from coupling with a 30-fold molar excess of DHT, in agreement with spectral measurements (*vide supra*). The results obtained with 4-, 12-, and 20-fold molar excesses of the reagent are analogous. The decrease in peptidase activity as well as the differences in the number of lysyl, tyrosyl, and histidyl residues, modified by 30-fold molar excess of DHT in the presence and absence of β-phenylpropionate, are correlated in Figure 7. In addition to tyrosine, the protected and unprotected enzymes differ by the coupling of one histidyl residue, and this modification seems closely related to the decrease in peptidase activity.

TABLE I: The Amino Acid Composition of Carboxypeptidase[a] Coupled with DHT in the Presence and Absence of β-Phenylpropionate.

Amino Acid	Native	Eightfold Molar Excess of DHT		30-fold Molar Excess of DHT	
		Unprotected	Protected[b]	Unprotected	Protected[b]
Lys	*14.8*	*11.2*	*11.0*	*3.1*	*2.9*
His	*7.6*	*7.4*	*7.4*	*6.7*	*7.6*
Arg	9.7	9.6	9.7	9.9	9.9
Asp	27.1	27.5	27.3	27.1	27.1
Thr	22.1	22.5	22.3	22.3	22.3
Ser	27.7	28.1	28.1	28.2	27.0
Glu	24.5	24.6	24.5	24.5	24.3
Pro	10.1	10.2	10.0	10.1	10.1
Gly	22.3	22.6	22.4	22.6	22.5
Ala	19	19	19	19	19
Val	14.6	14.9	14.4	14.5	14.5
Met	2.9	2.9	2.9	2.8	2.8
Ile	17.2	17.4	17.1	17.1	17.2
Leu	22.9	23.2	22.9	23.2	22.9
Tyr	*18.3*	*17.2*	*18.1*	*16.1*	*15.8*
Phe	14.7	14.6	14.6	14.9	14.6
Trp[c]	7.9	7.8	7.9	7.9	7.8

[a] Results are expressed as moles of amino acid per mole of enzyme and are calculated on the basis of 19 alanines/mole. [b] Coupled with DHT in the presence of 0.1 M β-phenylpropionate. [c] Colorimetric determination with *p*-dimethylaminobenzaldehyde (Spies and Chambers, 1949).

Successive Modification of Carboxypeptidase. To achieve greater selectivity in regard to the functional residue(s) affected by DHT, carboxypeptidase was succinylated and allowed to dialyze for 48 hr. During this period the *O*-succinyltyrosyl residues desuccinylate spontaneously, leaving the tyrosyl residues free to react, while 10 to 11 of the 15 ε-amino groups of lysine remain blocked (Riordan and Vallee, 1964). The product, *N*-succinylcarboxypeptidase, was then coupled with DHT in the standard manner. The resultant *N*-succinylazocarboxypeptidase exhibits enzymatic (Figure 8A) and spectral characteristics virtually indistinguishable from those of azocarboxypeptidase. Apparently modification of those lysyl residues which are accessible to succinylation is not responsible for the observed changes of activity. Furthermore, *N*-succinylazocarboxypeptidase retains its capacity to undergo additional functional changes when further modified chemically, as demonstrated by iodination. *N*-Succinylazocarboxypeptidase was dialyzed into 0.05 M Tris–1 M NaCl, pH 7.5, and iodinated with an eightfold molar excess of iodine monochloride (Simpson and Vallee, 1966), resulting in a fourfold increase of esterase activity; the remaining peptidase activity is less than 4% (Figure 8A).

A similar set of experiments involving DHT and *N*-acetylimidazole was performed but employing the reverse order. Carboxypeptidase was coupled with DHT and the product was acetylated with *N*-acetylimidazole. Esterase activity rises to that expected for acetylcarboxypeptidase (Figure 8B) (Simpson *et al.*, 1963; Riordan and Vallee, 1963). When peptidase activity of azocarboxypeptidase is present, it decreases to less than 3%.

This modification also was carried out in the order employed in the succinylation experiments. Acetylcarboxypeptidase containing 5.4 *O*-acetyltyrosyl residues and exhibiting 500% of the esterase and 8% of the peptidase activity of the control was coupled with a 30-fold molar excess of DHT at pH 8.8 for 10 min, 20°. The absorption of the product exhibiting a maximum at 480 mμ is quite similar to that observed on coupling of DHT with the native enzyme (Figure 3). The coupling reaction does not significantly alter the number of *O*-acetyltyrosyl residues (5.2), as determined by hydroxamate formation (Simpson *et al.*,

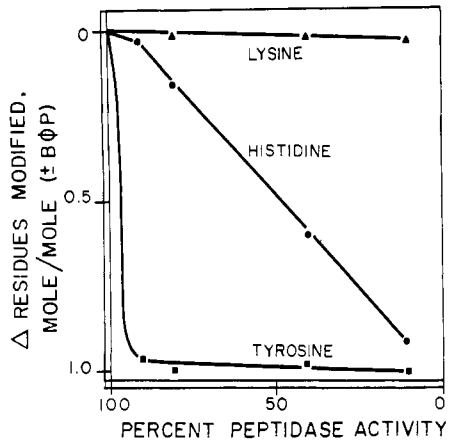

FIGURE 7: Decrease of peptidase activity of carboxypeptidase as a function of the number of lysyl, tyrosyl, and histidyl residues modified by molar excesses of DHT varying from 4- to 30-fold. The difference of the number of residues modified in the presence and absence of β-phenylpropionate is plotted.

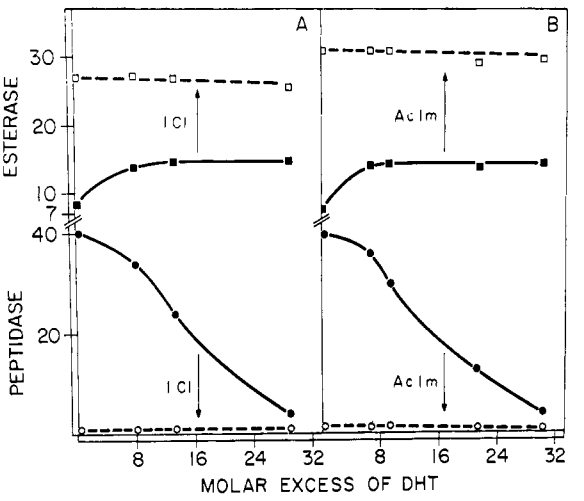

FIGURE 8: Triple (A) and double (B) chemical modifications of carboxypeptidase. (A) Native carboxypeptidase (10 mg/ml) was succinylated as described under Methods and dialyzed for 48 hr against 0.02 M Veronal–1 M NaCl buffer at pH 7.5; esterase activity, $k = 8.6 \times 10^3$, and peptidase activity, $C = 40$. The resultant *N*-succinylcarboxypeptidase (5×10^{-5} M) was then coupled with a molar excess of DHT varying from 1- to 30-fold, pH 8.8, 0°, for 30 min. The esterase (■—■) and peptidase (●—●) activities were then measured. The product, azo-*N*-succinylcarboxypeptidase, was dialyzed for 18 hr at 4° against 0.05 M Tris–1 M NaCl buffer, pH 7.5, and then iodinated with an eightfold molar excess of iodine monochloride for 5 min at 0°. The esterase (□—□) and peptidase activities (○—○) were then measured again as indicated by the arrows. (B) Carboxypeptidase (5×10^{-5} M) was coupled with a molar excess of DHT varying from 1- to 30-fold at pH 8.8, 0°, for 30 min; esterase (■—■) and peptidase (●—●) activities were measured. The enzyme was then dialyzed for 18 hr at 4° against 0.02 M Veronal–1 M NaCl, pH 7.5, and then acetylated with 200-fold molar excess of *N*-acetylimidazole, pH 7.5, for 30 min at room temperature as indicated by the arrows. Esterase (□—□) and peptidase (○—○) activities were then measured again. Assays were performed as described under Methods

1963). Since *O*-acetyltyrosine does not react with DHT (Sokolovsky and Vallee, 1966a), the increase in absorbance at 480 mμ would seem to be due only to the coupling of 0.91 histidyl residue.

The enzymatic changes of acetylcarboxypeptidase are fully reversed by incubation with 1 M hydroxylamine for 10 min at 25°, pH 7.5 (Simpson *et al.*, 1963). However, exposure of acetylazocarboxypeptidase to hydroxylamine restores only the esterase but not the peptidase activity (Table II).

TABLE II: Restoration of Activity to Various Derivatives of Carboxypeptidase by Deacetylation with Hydroxylamine.[a]

Enzyme	NH_2OH	Esterase ($k \times 10^3$)	Peptidase C
[(CPD)Zn]	0	7.1	39.9
[(AzoCPD)Zn]	0	10.2	3.6
[(AcCPD)Zn]	0	35.2	2.4
[(AcCPD)Zn]	+	6.6	37.2
[Azo(AcCPD)Zn]	+	5.8	7.9

[a] NH_2OH (1 M), pH 7.5, 10 min, room temperature.

The involvement of a specific amino acid residue in catalysis is generally inferred from loss of enzymatic activity owing to specific chemical modifications. Inferences concerning the involvement of particular residues are based on the assumption that inactivation is not attributable to nonspecific changes, *e.g.*, denaturation. Toward this end several chemically modified carboxypeptidases (Bethune *et al.*, 1964; Piras and Vallee, 1966) have been examined by a variety of methods including gel filtration. Azocarboxypeptidase (5×10^{-5} M) prepared with a 30-fold molar excess of DHT at 0–4° elutes from G-75 Sephadex (2 × 60 cm) as a single symmetrical peak and in the same position as the native enzyme. However, when higher concentrations of enzyme or of reagent are used, gel filtration reveals a major active and a minor inactive component. Therefore, a 30-fold molar excess was the maximum concentration of reagent used in these studies.

The pH–rate profile of esterase activity of azocarboxypeptidase differs from that of the native enzyme when both are assayed with 0.01 M HPLA. Above

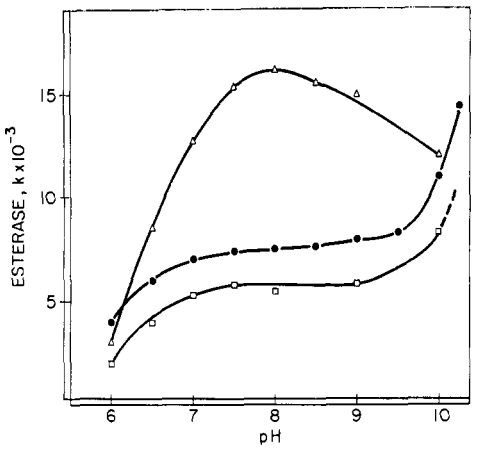

FIGURE 9: Esterase pH–rate profiles of native (●), azocarboxypeptidase (△), and azoacetylcarboxypeptidase deacylated with NH_2OH (□). Activities were measured with hippuryl-DL-β-phenyllactic acid as the substrate at the pH values indicated on the abscissa.

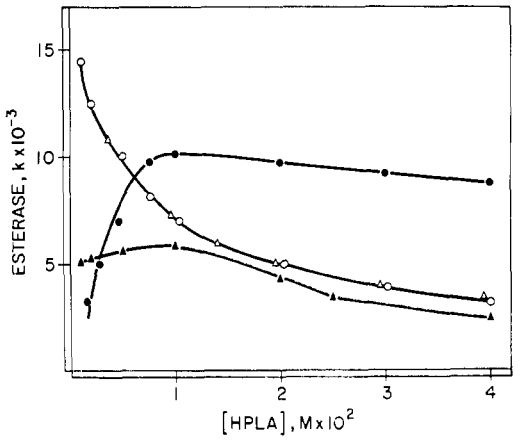

FIGURE 10: Esterase activities: substrate concentration dependence of azocarboxypeptidase (●), azocarboxypeptidase coupled in the presence of β-phenylpropionate (△), native carboxypeptidase (○), and azoacetylcarboxypeptidase deacetylated with 1 M hydroxylamine (▲). Assays were performed with hippuryl-DL-β-phenyllactic, pH 7.5.

pH 8.0 the rate of ester hydrolysis by the native enzyme increases while that of azocarboxypeptidase decreases (Figure 9). Coupling of DHT with acetylcarboxypeptidase followed by deacetylation with hydroxylamine does not change the pH dependence, however. The substrate concentration dependence of the modified enzyme differs from that of the native enzyme (Figure 10). Over the range of substrate concentration from 1×10^{-3} to 2×10^{-2} M the esterase activity of azocarboxypeptidase ($V_{max} = 1.5 \times 10^{-1}$ min^{-1}; $K_{m(app)} = 5.3 \times 10^{-3}$ M) increases as a function of substrate concentration while that of the native enzyme ($V_{max} = 2.8 \times 10^{4}$ min^{-1}; $K_{m(app)} = 8 \times 10^{-5}$ M) decreases with increasing substrate concentration. Although coupling in the presence of β-phenylpropionate modifies the enzyme extensively (Table I), the pH–rate profile and the substrate concentration dependence (Figure 10) coincide with those of the native enzyme (McClure *et al.*, 1964; Bender *et al.*, 1965).

Discussion

Coupling of diazonium salts with proteins has long been used to alter their immunological properties (Landsteiner, 1945). More recently, attention has been given to chemical modifications of specific amino acid residues with diazo compounds. Studies of chymotrypsin (Gundlach *et al.*, 1962) and bovine serum albumin (Tabachnik and Sobotka, 1960) indicate that these reagents couple almost exclusively with tyrosine, histidine, and lysine. Under appropriate conditions azo labels have been shown to be attached specifically to tyrosine residues at the active sites of antibodies (Metzger *et al.*, 1964). In agreement with previous studies employing similar agents on other proteins, tyrosine, lysine, and histidine were found to be modified here (Table I).

Horinishi and co-workers (1964) have recently examined diazonium-1H-tetrazole and have proposed its use as a coupling agent for histidyl residues, based on the spectral characteristics of its derivatives. In the course of evaluating the suitability of this reagent for the study of the active center of carboxypeptidase the formation both of mono- and bisazohistidyl and of mono- and bisazotyrosyl derivatives has been demonstrated, and their spectral properties have been reexamined (Sokolovsky and Vallee, 1966a). It was noted that the molar absorptivities of the azoamino acids, employed previously as standards of reference (Horinishi *et al.*, 1964), differ markedly from those of the corresponding *N*-acylazoamino acids. The characteristics of the latter are more nearly analogous to those of amino acid residues in proteins, of course. The spectra of the mono- and bisazotyrosyl and -histidyl derivatives have been determined and are here employed to evaluate the effect of the reagent on carboxypeptidase.

In using this reagent to identify a residue(s) involved in the mechanism of action of an enzyme, several considerations are important. Since monoazotyrosine and bisazohistidine both absorb maximally at 480 mμ, their relative contributions to the absorption at this wavelength must be differentiated. Further, a large excess of DHT results in the conversion of mono- to bisazotyrosine, which absorbs maximally at 548 mμ and also significantly at 480 mμ. The range of DHT concentration here employed, *i.e.*, 8–45-fold molar excesses over protein, largely precludes formation of bisazotyrosine.[4] While modifications with DHT alone

[4] Analysis of the carboxypeptidase data suggests that the absorption at 550 mμ would appear to be the result largely of absorption due to the monoazotyrosyl and bisazohistidyl rather than to the bisazotyrosyl chromophores.

do not yield unequivocal data implicating a specific residue, the reaction of low concentrations of DHT with carboxypeptidase considerably narrows the choice among the amino acid residues which might be responsible for enzymatic changes, particularly when amino acid analyses are performed simultaneously. The effects of protective agents, *e.g.*, inhibitors, substrates, and substrate analogs, can further focus upon the identity of critical residues. When other modifications of known chemical, spectral, and enzymatic consequences, *e.g.*, acylation or iodination, are combined with the coupling reaction, further discrimination becomes feasible.

The coupling of carboxypeptidase with DHT increases the rate of hydrolysis of the ester substrate, while decreasing peptidase activity under standard assay conditions as compared with the native enzyme. However, the effect of the coupling reaction on the activities of this enzyme differs significantly from those observed previously on acetylation and iodination (Vallee *et al.*, 1963) where the esterase and peptidase activities increase and decrease reciprocally. The maximal changes in esterase activity are induced by an eightfold molar excess of DHT which decreases peptidase activity only slightly (Figure 4). With increasing DHT concentrations, however, peptidase activity decreases progressively until, at a 45-fold molar excess, it becomes close to zero. The quantitative difference of the two activities in response to the varying concentrations of reagent suggests that the modification of different residues may account for these results. This supposition is supported by relating these changes to the increase in absorbance at 480 mμ, reflecting the formation of monoazotyrosyl and bisazohistidyl derivatives (Figure 6). At a point equivalent to the maximal changes in esterase activity, the molar absorptivity induced by the coupling reaction is 6000, corresponding either to 1.1 monoazotyrosyl or 0.3 bisazohistidyl residue. The subsequent increase in A_{480} is proportional to the decrease in peptidase activity, but esterase activity is not altered further. The changes in molar absorptivity of this derivative ϵ_{480} is 20,000 and would correspond to the coupling either of one histidyl or of four tyrosyl residues. Amino acid analysis reveals that, indeed, 0.9 histidyl residue is modified while maximally only 2.2 tyrosyl residues are coupled (Table I).

An eightfold molar excess of DHT coupled with carboxypeptidase modifies only one tyrosyl residue suggesting that the change in absorbance at 480 mμ and the coincident increase of esterase activity might be attributable to one monoazotyrosine rather than to 0.3 bisazohistidine (*vide infra*). The modification of the number of lysyl residues does not correlate with the changes in either one of the two activities, as indicated either by amino acid analysis (Figure 7), succinylation (Riordan and Vallee, 1964), acetylation (Simpson *et al.*, 1963), amidination, or succinylation followed by coupling. Azocarboxypeptidase contains 1 g-atom of zinc/mole of enzyme which protects the metal-binding sulfhydryl group from modification. Since under the present conditions DHT is shown to react only with tyrosine, lysine, and histidine, by elimination, the effect on peptidase activity would appear to be due to the modification of histidine.[5]

Sequential modification, *e.g.*, acetylation with *N*-acetylimidazole followed by coupling with DHT, further indicates that both tyrosyl and histidyl residues are involved. Acetylation shifts the maximum absorption of monoazotyrosine from 480 mμ to shorter wavelengths, but does not affect that of bisazohistidine, thereby differentiating between modifications of histidine and tyrosine. Thus, the decrease of absorption on acetylation of azocarboxypeptidase coupled with a 30-fold molar excess of DHT can be attributed to acetylation of 1.8 moles of monoazotyrosine while the remaining absorption corresponds to 0.92 mole of bisazohistidine/mole of protein.

Coupling followed either by acetylation with acetylimidazole or by iodination with iodine monochloride demonstrates that the active-center tyrosyl residues retain their potential to respond to acylation and iodination in enzymatically characteristic fashion even while coupled to DHT. Experiments employing the reverse sequence of modification, *e.g.*, acetylation followed by coupling, yield additional insight. Acetylation of the phenoxy group of tyrosyl residues prevents coupling with DHT. Thus, if the reaction of DHT with a tyrosyl residue would be responsible for the changes in peptidase activity, coupling of *O*-acetylcarboxypeptidase should not further alter its activity. Hence, acetylcarboxypeptidase was coupled with DHT and then deacetylated with hydroxylamine (Table II). As expected, esterase activity returns to a level closely similar to that of the native enzyme but, importantly, peptidase activity does not. This suggests that coupling with a residue other than tyrosine accounts for the sustained decrease of peptidase activity.

The esterase activities of native and azocarboxypeptidase differ in their pH dependence and are similar to, though not identical with, that of iodocarboxypeptidase (Simpson and Vallee, 1966). The esterase activity of azocarboxypeptidase, in which tyrosyl, histidyl, and lysyl residues are coupled, is maximal at pH 8 while that of the native enzyme is almost linear up to pH 9 and rises sharply thereafter (Vallee *et al.*, 1963). However, the pH profile of acetylazocarboxypeptidase, the tyrosyl residues of which are then deacylated with hydroxylamine (Figure 9), is quite similar to that of the native enzyme. One might conjecture that the differences in pH dependence between azo- and deacylated azoacetylcarboxypeptidase might reflect the altered pK of phenol ionization induced by coupling, analogous to that apparently

[5] It might be speculated that in the native enzyme tyrosine and histidine residues interact with one another. After coupling with tyrosine the histidyl residue might then become accessible, perhaps, owing to the changes in its microscopic, chemical environment. This hypothesis has been tested by performing the reactions as functions of pH and temperature. These efforts have been without success so far.

brought about by iodination (Simpson and Vallee, 1966); however, differences in the rate-limiting steps of ester hydrolysis for the native and modified enzymes cannot be excluded as the basis for this phenomenon. Removal of substrate inhibition accounts partially for the effects of coupling on activity, and further work will be required to detail the possible role of altered substrate binding and/or catalytic efficiency.

The present data suggest that in addition to tyrosine, a histidyl residue may be involved in the mechanism of action of carboxypeptidase. They do not indicate the manner of its participation, nor, in fact, do they eliminate the possibility that DHT may modify other residues which have not been identified as yet.

In conjunction with the results of previous chemical modifications, the present data raise some interesting questions. In all other instances studied thus far, alteration of both activities coincided with the modification of tyrosyl residues. The decrease of peptidase activity in the azoenzyme, however, appears related to the modification of a histidyl residue. A histidyl residue could not be shown thus far to account for the alterations of peptidase activity consequent to modifications by other agents. Hence, while the enzymatic end result is similar to previous ones, the manner in which it is brought about may differ. Thus, *e.g.*, the acetyl and iodo enzymes apparently no longer bind peptide substrates, such that they are hydrolyzed, presumably owing to the modification of one or two tyrosyl residues. The data presented here suggest that the histidine plays a role in either catalysis of peptide substrates, or binding, or both. Could this mean that there are different mechanisms of hydrolysis for ester and peptide substrates? It is difficult to answer this question on the basis of present evidence, but efforts, now in progress, to differentiate between these alternatives should prove revealing. On the basis of available data the enzymatic consequences of coupling with DHT might be viewed as a two-step reaction: coupling of a tyrosyl residue results in increased esterase activity and is followed by modification of a histidyl residue leading, in this instance, to an irreversible decrease in peptidase activity.

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References

Anson, M. L. (1937), *J. Gen. Physiol. 20*, 663, 777.

Bargetzi, J. P., Sampath Kumar, K. S. V., Cox, D. J., Walsh, K. A., and Neurath, H. (1963), *Biochemistry 2*, 1468.

Barnard, E. A., and Stein, W. D. (1958), *Advan. Enzymol. 20*, 51.

Barnard, E. A., and Stein, W. D. (1959), *Biochem. J. 71*, 19P.

Bender, M. L., Whitaker, J. R., and Menger, F. (1965), *Proc. Natl. Acad. Sci. U. S. 53*, 711.

Bethune, J. L., Ulmer, D. D., and Vallee, B. L. (1964), *Biochemistry 3*, 1764.

Coleman, J. E., and Vallee, B. L. (1960), *J. Biol. Chem. 235*, 390.

Coombs, T. L., Omote, Y., and Vallee, B. L. (1964), *Biochemistry 3*, 653.

Elkins-Kaufman, E., and Neurath, H. (1949), *J. Biol. Chem. 178*, 645.

Fuwa, K., and Vallee, B. L. (1963), *Anal. Chem. 35*, 492.

Gundlach, G., Kohne, C., and Turba, F. (1962), *Biochem. Z. 336*, 215.

Gundlach, H. G., Stein, W. H., and Moore, S. (1959), *J. Biol. Chem. 234*, 1754.

Horinishi, H., Hachimori, Y., Kurihara, K., and Shibata, K. (1964), *Biochim. Biophys. Acta 86*, 477.

Landsteiner, K. (1945), *in* the Specificity of Serological Reactions, Cambridge, Mass., Harvard Press.

McClure, W. O., Neurath, H., and Walsh, K. A. (1964), *Biochemistry 3*, 1897.

Metzger, H., Wofsy, L., and Singer, S. J. (1964), *Proc. Natl. Acad. Sci. U. S. 51*, 612.

Moore, S., and Stein, W. H. (1948), *J. Biol. Chem. 176*, 307.

Neurath, H., and Schwert, G. (1950), *Chem. Rev. 46*, 69.

Piras, R., and Vallee, B. L. (1966), *Biochemistry 5*, 855.

Riordan, J. F., and Vallee, B. L. (1963), *Biochemistry 2*, 1460.

Riordan, J. F., and Vallee, B. L. (1964), *Biochemistry 3*, 1768.

Simpson, R. T., Riordan, J. F., and Vallee, B. L. (1963), *Biochemistry 2*, 616.

Simpson, R. T., and Vallee, B. L. (1966), *Biochemistry 5*, 1760.

Snoke, J. E., Schwert, G. W., and Neurath, H. (1948), *J. Biol. Chem. 175*, 7.

Sokolovsky, M., and Vallee, B. L. (1966a), *Biochemistry 5*, 3574.

Sokolovsky, M., and Vallee, B. L. (1966b), 152nd National Meeting of the American Chemical Society, Abstracts, 171C.

Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.

Spies, J. R., and Chambers, D. C. (1949), *Anal. Chem. 21*, 1249.

Tabachnik, M., and Sobotka, H. (1960), *J. Biol. Chem. 235*, 1051.

Thiers, R. E. (1957), *Methods Biochem. Analy. 5*, 273.

Vallee, B. L., Riordan, J. F., and Coleman, J. E. (1963), *Proc. Natl. Acad. Sci. U. S. 49*, 109.