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Functional Tyrosyl Residues in the Active Center of Bovine Pancreatic Carboxypeptidase A*

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Received April 4, 1963

Acetylimidazole, a new agent for the acetylation of proteins, when reacted with carboxypeptidase A increases esterase activity and abolishes peptidase activity. These changes can be prevented by β-phenylpropionate and can be reversed by deacetylation with hydroxylamine. The alterations in enzymatic activities correlate exactly with the acetylation and deacetylation of two tyrosyl residues and are accompanied by spectral shifts which are attributable to the characteristic spectral properties of *O*-acetyltyrosine. Difference spectra and hydroxamate formation have been employed for a quantitative analysis of these changes.

The search for functionally active amino acid residues in the active center of carboxypeptidase A has recently received new direction by the results of chemical modifications of the enzyme. Acylation with monocarboxylic anhydrides, iodination, and photooxidation of the enzyme increase esterase and decrease peptidase activity (Riordan and Vallee, 1962; Vallee *et al.*, 1963). Extensions of these investigations using acetic anhydride (Riordan and Vallee, 1963; and in preparation), and iodine (Simpson and Vallee, 1963) have supported the view previously advanced (Vallee *et al.*, 1963), that tyrosyl or histidyl residues, or both, are involved in the catalytic mechanism of carboxypeptidase.

In the course of efforts to identify these groups, it has been found that acetylation of the enzyme with acetylimidazole also abolishes peptidase activity completely while esterase activity increases even more than with acetic anhydride. These alterations in specificity correlate closely to the acetylation of two tyrosyl residues, presumably located close to the catalytically active zinc binding site (Vallee *et al.*, 1960). The results with acetylimidazole show promise that this mild acetylating agent may prove more selective than monocarboxylic acid anhydrides. Acetylimidazole does not seem to have been employed previously as an agent for the acetylation of proteins.

Experimental

Materials.—Five times recrystallized carboxypeptidase A of bovine pancreas, [(CPD)Zn], was prepared by the method of Allan *et al.*[1] Enzyme prepared by the method of Anson (1937) was obtained from the Worthington Biochemical Corp., Freehold, New Jersey. The zinc-to-protein ratio of both preparations was between 0.98 and 1.03 g atoms/mole based on a molecular weight of 34,300 (Smith and Stockell, 1954; Vallee and Neurath, 1955; Brown *et al.*, 1961). *N*-acetylimidazole was prepared by the method of Boyer (1952), and when recrystallized from isopropenyl acetate had a melting range of 100–101°. *N*-acetyltyrosine and *N,O*-diacetyltyrosine were obtained from the Cyclo Chemical Corp., Los Angeles, and used without further purification. Precautions to prevent contamination by adventitious metal ions were taken throughout these studies (Thiers, 1957; Coleman and Vallee, 1960).

Methods.—*Peptidase activity* was determined using the synthetic substrate carbobenzoxyglycyl-L-phenylalanine (Mann Research Laboratories) (Coleman and Vallee, 1960). Activity is expressed as an apparent proteolytic coefficient, *C*, defined as $\log a_0/a$ per minute per μmole of enzyme, where a_0 and a represent the concentration of substrate at time zero and time t, respectively. The assays were carried out at 0° in 0.02 M sodium Veronal—1.0 M NaCl buffer, *p*H 7.5. *C* was calculated from the linear portion of the first-order reaction plots before hydrolysis exceeded 15%. *Esterase activity* was determined by *p*H titration (Snoke, *et al.*, 1948) with 0.1 M NaOH of the hydrogen ions released on hydrolysis using a *p*H-stat (Radiometer, Copenhagen) and recorder (Ole Dich, Copenhagen). Assays were performed at 25° with 5 ml of 0.01 M

* This work was supported by the Howard Hughes Medical Institute, by a grant-in-aid from the National Institutes of Health of the Department of Health, Education, and Welfare, No. HE-07297, and the Nutrition Foundation.

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[1] B. J. Allan, P. J. Keller, K. A. Walsh and H. Neurath, in preparation.

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 μmoles H^+ per minute per μmole of enzyme.

Protein concentrations were measured either by precipitation with 10% trichloroacetic acid followed by drying at 104° (Hoch and Vallee, 1953) or by absorbance at 278 mμ. The molar absorptivity of native carboxypeptidase A is 6.42×10^4 mole^{-1}cm^2, while the values employed for acetylcarboxypeptidase prepared with acetylimidazole, Ac_I-carboxypeptidase,[2] in the absence or presence of β-phenylpropionate were 6.17 and 5.9×10^4 mole^{-1}cm^2, respectively. A Beckman Model DU spectrophotometer was used for absorbance measurements at single wavelengths, and spectra were obtained with a Cary Model 15 MS automatic recording spectrophotometer. The pH was determined with a Radiometer pH meter (Model pHM4) equipped with a general purpose glass electrode. Acetylation of the enzyme with acetylimidazole was performed by adding a 10- to 100-fold excess of the reagent to a solution of carboxypeptidase (4 mg/ml) in 0.02 M sodium Veronal–2 M NaCl buffer, pH 7.5, at 23°. Reaction times varied from 2 to 120 minutes. The reaction was stopped either by dilution or by cooling and activities were determined immediately thereafter. Alternatively, the excess reagent was removed by exhaustive dialysis against 0.01 M Tris–1 M NaCl buffer, pH 7.5 at 4°. In some experiments, 0.05 M β-phenylpropionate was used as a protective agent (Riordan and Vallee, 1962). Labile acetyl groups were determined by the method of Balls and Wood (1956) using 1.14 M hydroxylamine at pH 7.5 and 25° for 10 minutes. The degree of modification of the free amino groups of carboxypeptidase was estimated by means of the ninhydrin reaction (Moore and Stein, 1954; Slobodian *et al.*, 1962) using phenylalanine as a standard.

Results

On reaction of carboxypeptidase with a 60-fold molar excess of acetylimidazole at pH 7.5, 23°, esterase activity increases to seven times the control value in 25 minutes. No further increase occurs if the reaction is allowed to continue. Peptidase activity is abolished completely (Fig. 1). As the molar excess of acetylimidazole is increased from 10- to 60-fold, the rate of acetylation increases also, and maximal changes in enzymatic activities are achieved at progressively earlier times. When higher molar proportions of acetylimidazole to protein are employed, esterase activity begins to decrease shortly after its maximum value is achieved; above a 100-fold molar excess of acetylimidazole, inactive enzyme precipitates rapidly (Fig. 2). Reaction with a 60-fold molar excess for 25 minutes at pH 7.5, 23°, was adopted as the standard procedure.

The competitive inhibitor β-phenylpropionate, at a concentration of 0.05 M, apparently *prevents* the acetylation of some specific groups of the protein since acetylation in its presence does not bring about the changes in catalytic specificity or activity (Fig. 1). Removal of β-phenylpropionate by dialysis, followed by acetylation under the standard conditions described, leads to a 7-fold increase in esterase and abolition of peptidase activity, changes identical to those obtained when the inhibitor is absent during the initial modification (Fig. 1).

[2] The prefixes Ac_A- and Ac_I- are used to designate carboxypeptidase acetylated with acetic anhydride and acetylimidazole, respectively.

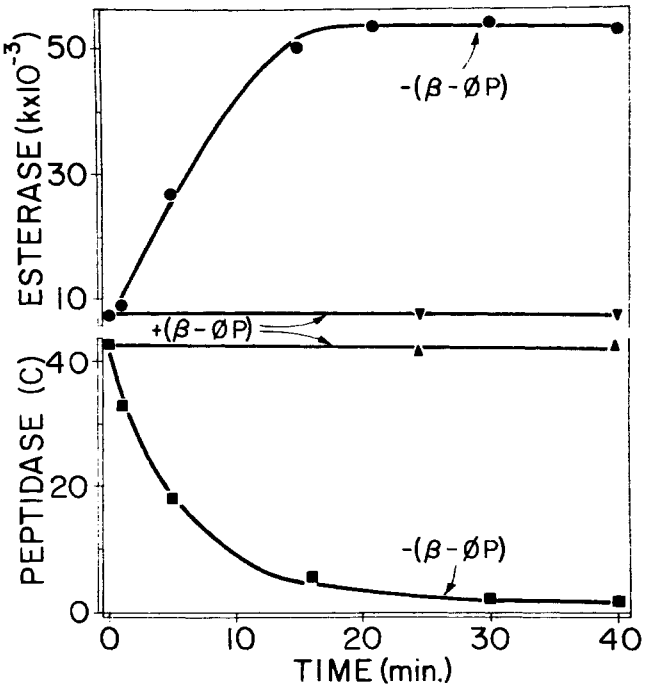

FIG. 1.—Progression of changes in esterase (●) and peptidase (■) activities during acetylation of carboxypeptidase (4 mg/ml) with acetylimidazole (60-fold M excess) and in the presence (▼, ▲) and absence of β-phenylpropionate, as indicated. The reactions were performed in 0.02 M sodium Veronal–2 M NaCl, pH 7.5, 23°. Activities were determined immediately following dilution of an aliquot of the reaction mixture into the same buffer. Acetylation in the presence of β-phenylpropionate does not change the activities of the native enzyme and serve as control.

Exposure of Ac_I-carboxypeptidase to hydroxylamine completely *reverses* these enzymatic changes. When 5×10^{-5} M Ac_I-carboxypeptidase is incubated with 1.14 M hydroxylamine at pH 7.5, 25°, for 10 minutes, peptidase and esterase activities are restored to the values characteristic of the native enzyme (Table I). Upon deacetylation with this reagent, esterase activity decreases from 710% to 100% while peptidase activity increases from 7% to 100% of the control (Table I). No alterations in functional properties are observed when the enzyme acetylated in the presence of β-phenylpropionate, or the native enzyme, is treated with hydroxylamine.

The enzymatic changes occurring on acetylation with acetylimidazole are virtually identical to those observed when carboxypeptidase is modified with acetic anhydride, though acetylimidazole does not acetylate NH_2

TABLE I

ACETYLATION AND DEACETYLATION OF Ac_I- AND Ac_A-CARBOXYPEPTIDASE

Samples were acetylated in the presence of β-phenylpropionate (βφP), 0.05 M, as indicated. Deacetylations with hydroxylamine, 1.14 M, were performed as under Methods.

[(CPD) Zn]	Esterase $k \times 10^{-3}$	Peptidase C	Eq. Phenylalanine / Mole Enzyme
Control	7.3	38.2	13.8 ± 0.6
Ac_I	52.0	3.0	13.9 ± 0.4
Ac_A	36.9	1.9	4.9 ± 0.9
Ac_I + βφP	7.5	39.1	13.1 ± 0.7
Ac_A + βφP	7.3	34.3	4.6 ± 0.5
Ac_I + NH_2OH	7.5	37.5	13.4 ± 0.1
Ac_A + NH_2OH	6.6	32.9	4.1 ± 0.5

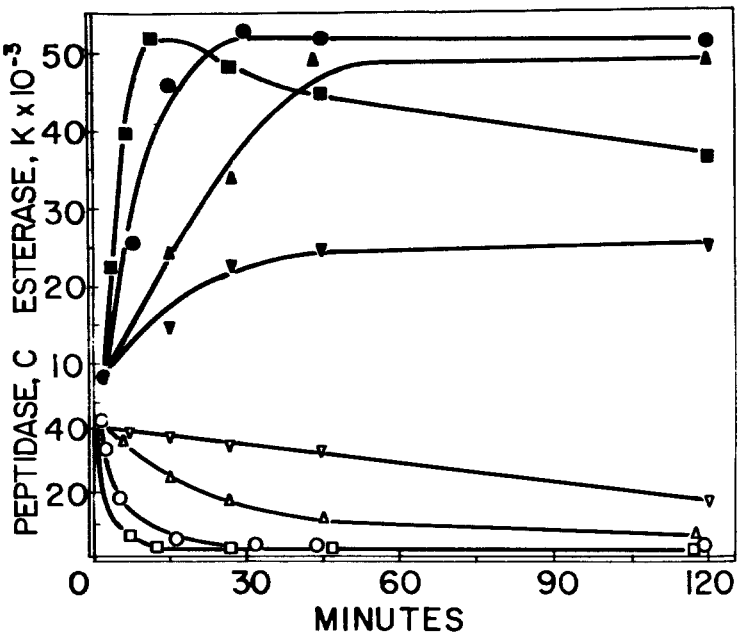

FIG. 2.—Acetylation of carboxypeptidase. Effects of variation of the molar excess of acetylimidazole on esterase and peptidase activities. Esterase (solid symbols) and peptidase (open symbols) were measured when the enzyme (4 mg/ml) was exposed to 10- (▼, ▽), 25- (▲, △), 60- (●, ○), and 100-fold (■, □) molar excess for the periods indicated. Conditions for acetylation and assay as in Figure 1.

groups, as judged by the procedure of Moore and Stein (1954) used according to Slobodian *et al.* (1962) and from electrophoretic mobility (Bethune and Ulmer 1963) (Table I). There are 13.8 ± 0.6 (n = 30) phenylalanine equivalents per mole of native enzyme and 13.9 ± 0.4 (n = 14) groups per mole of Ac_I-carboxypeptidase. In contrast, the phenylalanine equivalents decreased to 4.9 ± 0.9 (n = 23) for Ac_A-carboxypeptidase, the enzyme acetylated with acetic anhydride (Table I). Though acetylation with either reagent in the presence of β-phenylpropionate does not alter either esterase or peptidase activities (Fig. 1), the change in phenylalanine equivalents is virtually the same as in the absence of this protective agent, and deacetylation with hydroxylamine does not alter this value (Table I). Such data support the previous suggestion that the acetylation of ε amino groups does not account for the reversible alterations of activity (Vallee *et al.*, 1963).

The chemical nature of the residues involved in the catalytic action of carboxypeptidase have been the subject of speculation (Vallee *et al.*, 1963) but their identity has been unknown. The close correlation between spectral and catalytic changes of the enzyme on acetylation now indicates the participation of tyrosyl residues either in the mechanism of hydrolysis or in substrate binding to the enzyme, or both. Relative to the spectrum of native carboxypeptidase between 260 and 300 mμ, the absorbance of Ac_I-carboxypeptidase decreases (Figs. 3A and 3C). The difference spectrum has a maximum decrease at 278 mμ, suggesting an alteration in the aromatic amino acid residues, presumably tyrosine (Fig. 3C).

The spectrum of tyrosine, when contrasted with that of *N,O*-diacetyltyrosine, supports this inference (Figs. 3B and 3D): *N*-acetyltyrosine absorbs maximally at 275 mμ. The molar absorptivities at *p*H 7.5 are ϵ_{275} = 1370 and ϵ_{262} = 620. The maximal absorbance of *N,O*-diacetyltyrosine is shifted from 275 to 262 mμ (Fig. 3B), and the molar absorptivities at *both* wavelengths are lowered markedly, ϵ_{275} = 95 and ϵ_{262} = 280. The spectrum of *N,O*-diacetyltyrosine closely resembles that of phenylalanine in this wavelength range (Fig. 3B). The spectrum of this tyrosine derivative in 0.1 N HCl has been reported (Schlögl, 1953).

The difference spectrum of *N*-acetyltyrosine versus *N,O*-diacetyltyrosine in Figure 3D accentuates the magnitude of this spectral change on *O*-acetylation. The similarity of this difference spectrum to that of native carboxypeptidase versus Ac_I-carboxypeptidase suggests that the acetylation of tyrosyl residues of the enzyme accounts for the observed spectral changes.

Hydroxylamine reverses the enzymatic consequences of acetylation of carboxypeptidase with acetic anhydride (Riordan and Vallee, 1962; Vallee *et al.*, 1963) and with acetylimidazole (Table I). Similarly, deacetylation of *N,O*-diacetyltyrosine with hydroxylamine restores the spectrum of *N*-acetyltyrosine. To ensure precision, this change is measured at a wavelength where the difference in molar absorptivity is maximal. The maximum of the difference spectrum of the tyrosine derivatives is at 275 mμ while that of the native versus Ac_I-carboxypeptidase occurs at 278 mμ. Exposure of Ac_I-carboxypeptidase to 0.01 M NH_2OH for 450 minutes at *p*H 7.5, room temperature, fully restores the spectrum of native carboxypeptidase. Simultaneously esterase and peptidase activities return to the values characteristic of the native enzyme.

The difference in molar absorptivity of *N,O*-diacetyltyrosine and of *N*-acetyltyrosine may be used to calculate the number of acetyl groups removed from Ac_I-carboxypeptidase by hydroxylamine. At 278 mμ, the wavelength of maximal change in the difference spectrum of the enzyme, the molar absorptivity of *N,O*-diacetyltyrosine, ϵ'_{278}, rises from 70 to 1230 on conversion to *N*-acetyltyrosine. This corresponds to a difference, $\Delta\epsilon'_{278}$, of 1160 per mole of *N,O*-diacetyltyrosine deacetylated. Hence the change in absorbancy

for the transition of Ac_1-carboxypeptidase to native carboxypeptidase can be expressed by equation (1):

$$n = \frac{\Delta\epsilon_{278}}{\Delta\epsilon'_{278}} \quad (1)$$

where n is the number of *O*-acetyltyrosyl groups deacetylated, $\Delta\epsilon_{278}$ is the change in the molar absorptivity of the enzyme at 278 mμ on deacetylation, and $\Delta\epsilon'_{278}$ is the change in molar absorptivity at 278 mμ for the transition of *N,O*-diacetyl- to *N*-acetyltyrosine. Substituting the experimental results in equation (1) gives an average value of 4.3 tyrosyl groups acetylated.

If the formation of *O*-acetyltyrosyl residues is in any way responsible for the catalytic consequences of acetylation, the number of these residues should differ in β-phenylpropionate-protected and unprotected Ac_1-carboxypeptidase preparations. The enzyme, therefore, was acetylated with acetylimidazole both in the presence and absence of inhibitor. Subsequent to the removal of excess reagent and inhibitor by dialysis, both preparations were exposed to 0.01 M NH_2OH, at *p*H 7.5, 23°. The rates of changes in spectra and of enzymatic rates of the two preparations were then compared. Deacetylation of the unprotected enzyme has a much more rapid initial rate than that of the protected enzyme. After 110 minutes, however, their rates of change are virtually identical (Fig. 4); moreover, at this time the enzymatic rates are restored to the values characteristic of the native enzyme. Subtraction of the rate of deacetylation of the protected control from that of the unprotected enzyme yields the rate for the preferentially deacetylated residues (Fig. 4). Based on the change of molar absorptivity during the deacetylation of *N,O*-diacetyltyrosine, the data presented in Figure 4 indicate that β-phenylpropionate protects two tyrosyl residues. Four separate experiments indicate that 2 ± 0.05 tyrosyl residues are deacetylated.

Figure 5 demonstrates striking correlation between the rates of spectral change and the return of peptidase and esterase activities to those characteristic of native carboxypeptidase. Further, carboxypeptidase acetylated in the presence and absence of β-phenylpropionate was exposed to 1.14 M hydroxylamine, and the number of acetyl groups removed from both preparations was determined by measuring the number of moles of acethydroxamate formed by the procedure of Balls and Wood (1956). Ten determinations show a difference of 2.1 ± 0.25 acetyl groups between the protected and unprotected preparations.

Discussion

The competitive inhibitor β-phenylpropionate prevents the enzymatic changes resulting from acetylation with acetylimidazole. It does so, apparently, by protecting approximately two tyrosyl groups of carboxypeptidase. The rate of restoration of the enzymatic properties to those characteristic of the native enzyme is virtually identical with the rate of deacetylation of these tyrosyl residues, suggesting their participation in the catalytic mechanism of carboxypeptidase. The two tyrosyl residues protected by β-phenylpropionate and by other competitive inhibitors of the enzyme[3] (Riordan and Vallee, in preparation) are those which are deacetylated preferentially with hydroxylamine.

The marked difference in the rate of deacetylation of these two tyrosyl residues might be compared with the interaction of certain reactive amino acid groups with site specific, selective reagents. A unique seryl residue, for example, is known to react specifically with diisopropylfluorophosphate in chymotrypsin (Balls and Aldrich, 1955) and acetylcholine esterase (Wilson, 1960). Similarly the ε-amino group

[3] The changes in activities which occur on treatment with acetic anhydride can be prevented by β-phenylpropionate, indoleacetate, iodoacetate (all 0.1 M), and by half-saturated sodium acetate.

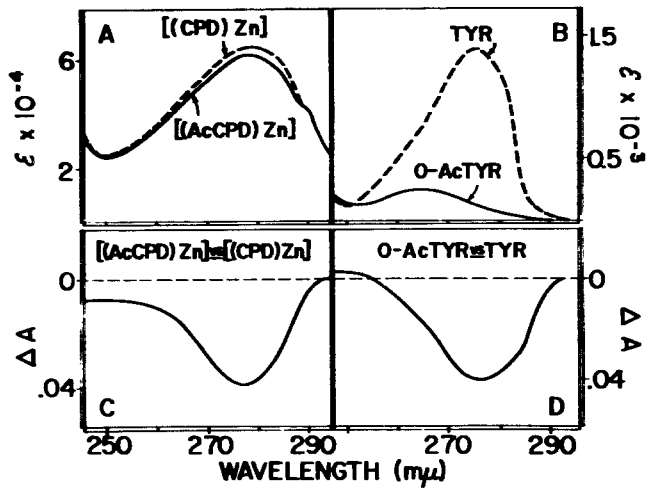

FIG. 3.—Absorption spectra of carboxypeptidase and Ac_1-carboxypeptidase (A), and tyrosine (Tyr)* and *N,O*-diacetyltyrosine (*O*-AcTyr) (B). Difference spectra of Ac_1- and native carboxypeptidase (C), and tyrosine* and *N,O*-diacetyltyrosine (D). The protein concentration in A and the amino acid concentrations in B were identical for each pair. The concentration in C was 7.5×10^{-6} M and in D was 3.2×10^{-5} M. Cary recording spectrophotometer Model 15, 1-cm. cell. All spectra were obtained in 0.01 M Tris–1 M NaCl buffer at *p*H 7.5, 23°.

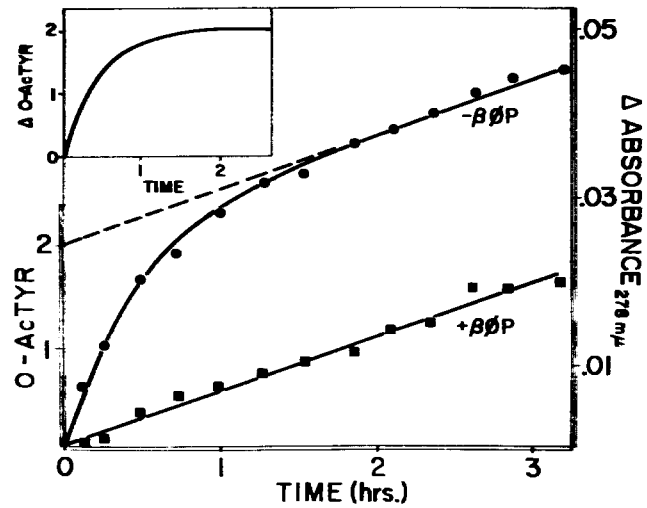

FIG. 4.—Deacetylation of Ac_1-carboxypeptidase prepared by exposure of 60 M excess of acetylimidazole for 25 minutes, *p*H 7.5, 23°, then 0.01 M hydroxylamine for the periods indicated. Aliquots of the same concentration of enzyme (1.1×10^{-6} M) were acetylated in the presence (■) and absence (●) of β-phenylpropionate, 0.05 M, as labeled, and their change in absorbance was then followed at 278 mμ (right ordinate). The left ordinate shows the number of the *O*-acetyltyrosyl groups deacetylated, as calculated from equation (1), (see text). The dashed line represents an extrapolation of the linear portion of the rate of deacetylation of the enzyme acetylated without β-phenylpropionate. The insert details the time course of the *difference* between the two rates of deacetylation.

* The spectra of tyrosine and *N*-acetyltyrosine are superimposable.

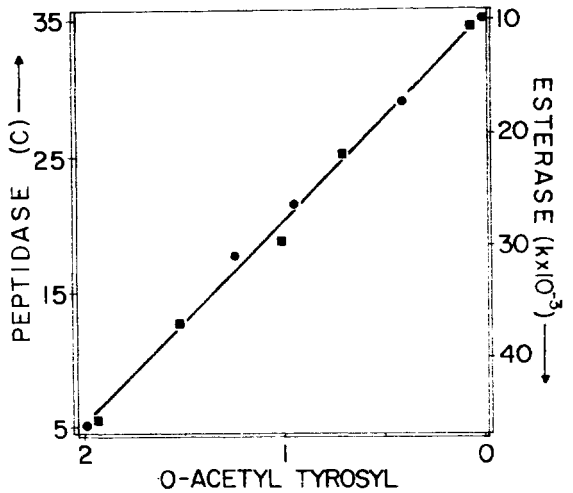

FIG. 5.—Correlation between the effects of hydroxylamine on peptidase (■) and esterase (●) activities and numbers of *O*-acetyl groups removed from Ac_I-carboxypeptidase. The enzyme acetylated with 60-fold molar excess of acetylimidazole under standard conditions (see methods) was exposed to 0.01 M hydroxylamine, *p*H 7.5, 23°, and the changes in peptidase and esterase activities and absorbance at 278 mμ were measured over a period of 110 minutes. The control was an aliquot acetylated in the presence of β-phenylpropionate. A K value of 7–8 × 10^3 and C values of 35–40 were characteristic both of the native enzyme and of that acetylated in the presence of the inhibitor in these experiments (see Fig. 1). Note the *direction* of change in esterase and peptidase activities as indicated by the arrows along the ordinates.

of the lysyl residue at position 41 (Hirs *et al.*, 1962) of bovine pancreatic ribonuclease is known to possess special activity toward fluorodinitrobenzene and *N*-carboxy-DL-alanine anhydride (Cooke *et al.*, 1963). In carboxypeptidase, the presence or absence of 4 M guanidine at *p*H 7.5 does not alter the number of acetyl groups transferred to hydroxylamine, consistent with the behavior of *N,O*-diacetyltyrosine in 8 M urea as reported by Cohen and Erlanger (1961). This lends support to the conclusion that acetylation of tyrosyl residues accounts both for the spectral and enzymatic changes.

Together with studies to be reported elsewhere (Simpson and Vallee, 1963), the present investigation suggests that the nineteen tyrosyl residues of carboxypeptidase (Bargetzi, *et al.*, in preparation) may be divided into three categories. Two of these are differentiated by their hydrogen ion titration curves, and the third category by the susceptibility of its acetyl derivatives to deacetylation with hydroxylamine.

Seven of the nineteen tyrosyl residues of carboxypeptidase dissociate "normally." Their apparent pK_a is 9.5, and their dissociation is complete at *p*H 10.2. The remaining twelve residues dissociate "abnormally" and are titrated above *p*H 10.5 (Simpson and Vallee, 1963). Hence they may be "buried" in the interior of the native protein, and, by analogy with other proteins (Tanford, 1961; Scheraga and Rupley, 1962), their failure to dissociate at lower *p*H may be a reflection of their role in stabilizing the conformation of the molecule.

The spectral changes indicate that only a limited number of tyrosyl residues are acetylated under the conditions of these experiments (Fig. 3). While the total number of groups acetylated with acetylimidazole remains to be determined by more direct methods such as amino acid analysis or radioactive labeling, the number of acetyl groups may be calculated from the spectral data (Fig. 4) as follows: the molar absorptivity of carboxypeptidase, ϵ_{278}, is 6.42 × 10^4 $mole^{-1}$ cm^2, determined by the trichloroacetic acid procedure of Hoch and Vallee (1953). The molar absorptivity of Ac_I-carboxypeptidase, ϵ_{278}, is 5.92 × 10^4 $mole^{-1}$ cm^2, changing rapidly to 6.15 × 10^4 $mole^{-1}$ cm^2, and finally to 6.42 × 10^4 $mole^{-1}$ cm^2, the value of the native enzyme observed initially, on exposure to hydroxylamine. The molar absorptivity of *N,O*-diacetyltyrosine increases by 0.116 × 10^4 $mole^{-1}$ cm^2 at 278 mμ upon deacetylation. Based on this, the change in the molar absorptivity of Ac_I-carboxypeptidase, 0.50, indicates that a total of 4.3 tyrosyl groups are deacetylated, two of which are especially susceptible to hydroxylamine and apparently responsible for the changes in activity as demonstrated by the correlation plot of Figure 5. Analogous data with Ac_A-carboxypeptidase have been obtained also indicating the correlation between the deacetylation of two tyrosyl residues and the restoration of function (Riordan and Vallee, 1963).

It would be reasonable to assume that exposed tyrosyl residues, presumably on the "surface" of the molecule, would be acetylated more readily than those in its interior. Acetylimidazole may well acetylate 4–5 of those residues which dissociate "normally." At comparable molar excesses, acetic anhydride appears to acetylate a total of 6–7 (Riordan and Vallee, in preparation) and 6 residues are iodinated with a 25-fold molar excess of iodine at *p*H 9, the number varying somewhat with the molar excess of iodine and the conditions employed (Simpson and Vallee, 1963).

Apparently the detection of the two tyrosyl residues, which are both readily acetylated and then particularly susceptible to deacetylation, is facilitated by their physicochemical properties, made unique by their molecular environment. The zinc atom which is involved in catalysis is bound to cysteine (Vallee *et al.*, 1960) and to the NH_2-terminal asparagine residue of the enzyme (Coombs and Omote, 1962). In this regard it should be noted that tyrosine is the penultimate NH_2-terminal residue (Coombs and Omote, 1962); moreover tyrosine is also present in the peptide which contains the metal-binding cysteine residue (Neurath, H., personal communication). The increase in esterase activity of Ac_I-carboxypeptidase is slightly greater than that of Ac_A-carboxypeptidase (Riordan and Vallee, 1962), perhaps the consequence of the more selective action of acetylimidazole. Molar excesses of acetic anhydride above 48 result in irreversible losses of function and losses of zinc (Riordan and Vallee, 1963), and are accompanied by structural changes in the molecule, as indicated by optical rotatory dispersion data and by the isolation of an enzymatically inactive fraction by free boundary electrophoresis (Bethune and Ulmer, 1963). The structural changes of Ac_I-carboxypeptidase, when prepared with a comparable molar excess of acetylimidazole, are much less marked, while the functional changes are even greater (Fig. 2). Preliminary electrophoretic and optical rotatory dispersion measurements give little evidence of structural change of Ac_I-carboxypeptidase, prepared with a 60-fold molar excess of the agent at *p*H 7.5 (Bethune *et al.*, in preparation). Further, there is no change in the number of free amino groups when carboxypeptidase is acetylated with acetylimidazole in contrast to preparations acetylated with acetic anhydride (Table I).

The conclusions reached in the present investigation have depended greatly on the changes in the absorption spectrum of carboxypeptidase. The interpretation of difference spectra here employed, the technical and instrumental limitations of the procedure, and examples

of its increasingly frequent use for the identification of catalytically active residues of enzymes have been the subjects of an excellent and extensive recent review (Wetlaufer, 1962). It would seem unnecessary, therefore, to detail these problems here. Many of the ambiguities inherent in the interpretations of the spectra of most amino acid residues, which absorb only at low wavelengths, do not exist in the case of tyrosyl residues. This circumstance has greatly facilitated the present studies. The spectral properties of tyrosyls in proteins have been detailed (Beaven and Holiday, 1952; Edsall and Wyman, 1958; Tanford, 1961). Both the spectral changes which are due to the ionization of tyrosine and the pertinent microscopic dissociation constants have been described, and the possible influences of solvents, temperature, conformation and "vicinal" effects on tyrosine spectra have been considered (Wetlaufer, 1962).

The marked hypo- and hypsochromic effects of acetylation on the spectrum of *N*-acetyltyrosine noted here have been recorded previously for the acetylation of tyrosine but have been studied under somewhat different conditions (Schlögl *et al.*, 1953). These changes are not subject to confusion with the hyper- and bathochromic effects of ionization or iodination of tyrosine (Martin *et al.*, 1958; Edelhoch, 1962). The marked changes in molar absorptivity on acetylation are greater by an order of magnitude than those to be expected from "vicinal" effects (Wetlaufer, 1962). The changes seen here are analogous to the spectral shifts observed when phenol is acetylated to form *O*-acetylphenol, and they are in clear contrast to the results of methylation of tyrosine to form *O*-methyltyrosine (Wetlaufer *et al.*, 1958).

The spectral shift in carboxypeptidase is analogous, however, to that observed for tyrosine-*O*-sulfate (Bettelheim, 1954, Dodgson *et al.*, 1959.) In contrast to methylation, acylation[4] or arylation apparently induce analogous electronic shifts in tyrosyl groups of proteins, as exemplified by the tyrosyl-*O*-sulfate residue of native fibrinogen and the *O*-acetyl groups of acetylcarboxypeptidase. Trypsin and chymotrypsin also exhibit similar changes in their absorbance when they are acetylated with acetylimidazole (Riordan, Wacker, and Vallee, unpublished data). It should be noted that the amidation of the free carboxyl function does not change the spectrum of *N*-acetyltyrosine significantly.

Studies of pepsin (Herriott, 1935) led early to suggestions that tyrosyl residues might be involved in the catalytic mechanism of this enzyme. Since then their role in the mechanism of other enzymes has also been postulated though experimental evidence has been slow to develop.

The present work does not attempt to discern the manner in which the tyrosyl residues participate in the mechanism of carboxypeptidase action. A model, proposed recently, assumes that the modification of one amino acid residue is the simplest chemical event which could account for the increased esterase and decreased peptidase activities which are the enzymatic consequences of many of the chemical alterations of this enzyme (Vallee *et al.*, 1963). Without experimental evidence for its existence, a second group was also postulated to participate in peptide hydrolysis, a speculation which is not inconsistent with the present findings.

[4] We are indebted to Dr. Abraham Patchornik for communicating to us, prior to publication, spectral data for *O*-acetyltyrosine and *O*-carbobenzoxytyrosine. The spectral properties of his compounds and those here employed were identical.

The characteristics of the peptidase *p*H rate profile, the dependence of the enzymatic consequences of acetylation of the enzyme on the values of *p*H at which the modification is performed, and the results of photooxidation have all suggested that a histidyl residue may also be involved in activity (Vallee *et al.*, 1963; Riordan and Vallee, in preparation). The difference spectra in the range 235–250 mμ of Ac_A-of Ac_I-carboxypeptidase versus carboxypeptidase do not indicate the formation of an acetylhistidyl residue. The deacetylation of the enzyme with hydroxylamine at near neutral *p*H further militates against an *N*-acetyl bond. Nevertheless, the previous findings pointing to histidine (Vallee *et al.*, 1963) require further consideration that this or additional amino acid residues might be involved in the catalytic mechanism. Nor can the real possibility be excluded that acetylation or other chemical modifications of carboxypeptidase affect esterase and peptidase activities—partially or completely—through the modification of group(s) at the enzyme surface which bind the different substrates.

The further exploration of these problems, together with kinetic investigations, now in progress, should aid materially in the elucidation of the catalytic mechanism of carboxypeptidase. The study of the possibility that tyrosyl residues may also be involved in the mechanism of action of other enzymes may benefit from the approaches here detailed.

Acknowledgment

We are indebted to Dora Saunders and Janet Carlson for valuable technical assistance.

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