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Organic Modifications of Metallocarboxypeptidases*

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ABSTRACT: Both acetylation and iodination of tyrosyl residues and substitution of other metal ions for the native zinc atom alter the catalytic specificity of carboxypeptidase A. Both types of modification now have been employed jointly to explore further the intramolecular relationship between the catalytically essential metal atom and the functional tyrosines. The studies demonstrate that while the enzymatic consequences of such different chemical reactions are similar the mechanisms which bring them about are not. Acetylation or iodination of zinc carboxypeptidase abolishes peptidase activity through curtailment of the binding of synthetic dipeptide substrates such as carbobenzoxyglycyl-L-phenylalanine. Deacetylation of tyrosyl groups with hydroxylamine restores both binding and peptidase activity. However, both acetyl- or iodocarboxypeptidase bind the ester hippuryl-*dl*-β-phenyllactate.

The enzymatic consequences of metal substitution conjointly with acetylation or iodination of carboxypeptidase are not additive. The esterase activities of zinc, cadmium, cobalt, nickel, and manganese acetyl- and iodocarboxypeptidases are predominantly those characteristic of the respective metals. The peptidase activities, however, are those expected on acetylation or iodination of tyrosine. The esterase activity of mercury carboxypeptidase is comparable to that of the native zinc enzyme; but the mercury enzyme does not hydrolyze peptides even though these substrates are bound. On acetylation or iodination the esterase activity of mercury carboxypeptidase is abolished even though the ester substrate hippuryl-*dl*-β-phenyllactate continues to be bound. Further, under appropriate conditions, Zn^{2+} replaces mercury; the esterase activities then return to those of acetyl or iodo zinc carboxypeptidase indicating the distinctive but interdependent contribution both of the metal ion and of modification of amino acid residues to the enzymatic process.

A number of metallocarboxypeptidases have been prepared by replacing zinc with a series of other metal ions. When assayed under identical conditions these metalloenzymes differ from the native enzyme in specific activity and in some cases even in apparent substrate specificity. These replacement studies have presented a means to explore the role of the metal in the mechanism of action of carboxypeptidase and have also assisted in the delineation of the metal-binding site (Vallee *et al.*, 1960a,b; Coleman and Vallee, 1961; Vallee *et al.*, 1961). Acetylation and iodination of carboxypeptidase also has major functional consequences due to modification of two of the 19 tyrosyl residues of the enzyme (Riordan and Vallee, 1963; Simpson *et al.*, 1963; Simpson and Vallee, 1966).

A working hypothesis which invoked the operation of a minimal number of groups resulted in a scheme which attempted to attribute all functional changes to the modification of a single residue. The resultant model was proposed in order to generate and test alternate hypotheses concerning the possible mechanisms of action of the enzyme. The model neither assigned any role to the specificity-determining C-terminal carboxyl group of the substrate nor could it take into account possible effects of modification on substrate binding, since only minimal information on these

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features was then available (Vallee *et al.*, 1963). The discovery of the functional changes accompanying the chemical modification of two tyrosyl residues *subsequent* to this formulation extended the scope of previous interpretation and afforded opportunities for new experimental approaches (Riordan and Vallee, 1963). The joint organic and inorganic modifications which can now be performed give rise to a total of over 500 permutations, which may exhibit characteristic functional properties (Vallee, 1964a,b). From this large number of derivatives a certain few have been selected for study to gain further insight into the mechanism of action of carboxypeptidase, and, if possible, to assign roles to the metal atoms and tyrosyl residues in substrate binding and catalysis or both. Concurrently, detailed kinetic investigations of certain derivatives have been undertaken (Riordan *et al.*, 1965a). The detailed results of these studies will be the subject of separate communications.

Peptidase activity of carboxypeptidase is virtually lost by replacing Zn^{2+} with Cd^{2+}, Hg^{2+}, or alternatively, by acylation or iodination of the native enzyme. The resultant derivatives are all active esterases (Vallee, 1964a). The experiments to be reported were designed to discern whether or not the loss of peptidase and increase in esterase activities achieved by these chemically different approaches could be reduced to a common denominator. The results of combined modifications to be reported here demonstrate that while the *enzymatic consequences* of such different chemical reactions are similar, the *mechanisms* which bring them about are not. Preliminary reports have been rendered (Coleman *et al.*, 1964; Vallee, 1964b).

Experimental Procedures

Beef pancreas carboxypeptidase, [(CPD)Zn],[1] was prepared and recrystallized four times by the method of Allan *et al.* (1964). The zinc/protein ratio of the preparation was between 0.98 and 1.03 g-atom/mole based on a molecular weight of 34,600 (Brown *et al.*, 1961).

Metal-free apocarboxypeptidase (CPD) and Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Cd^{2+}, and Hg^{2+} Carboxypeptidases were prepared as previously described (Coleman and Vallee, 1961, 1962a).

Acetylcarboxypeptidase was prepared by acetylation of the enzyme with *N*-acetylimidazole (Simpson *et al.*, 1963). The reaction was performed by adding a 60-fold molar excess of the reagent to 10^{-4} M solutions of carboxypeptidase or a 100-fold molar excess to 10^{-5} M solutions of the enzyme in 0.02 M sodium Veronal–1.0 M NaCl buffer, pH 7.5, at 23°. A greater excess was used for the lower concentration of enzyme since under these conditions the relative rate of spontaneous decomposition of *N*-acetylimidazole is greater. The time course of the reaction of different metallocarboxypeptidases was followed for 180 min. The reaction was stopped either by dilution or by dialysis *vs.* 0.02 M sodium Veronal–1.0 M NaCl, pH 7.5, 4°.

Iodination was performed with iodine monochloride prepared according to the procedure of McFarlane (1958) as modified by Koshland *et al.* (1963) (Simpson and Vallee, 1966). A small amount of the ICl solution containing an eightfold molar excess of ICl over enzyme was added to a carboxypeptidase solution (3–6 mg/ml) in 0.05 M Tris–1.0 M NaCl, pH 7.5, 4°. After 10 min of reaction the enzyme was dialyzed *vs.* 0.05 M Tris–1.0 M NaCl, pH 7.5, 4°.

Acetyl- and iodoapocarboxypeptidases were prepared by removal of the zinc with 1,10-phenanthroline as described for the native enzyme (Vallee *et al.*, 1960). The zinc content varied from 27 to 86 μg/g of protein or 1.4–4.4% of the 1850–1950 μg of zinc originally present/g of the native enzyme.

Acetyl- and iodometallocarboxypeptidases were obtained either by first preparing a given metallocarboxypeptidase which was then acetylated or iodinated at pH 8 or first by iodinating or acetylating the zinc enzyme at pH 7.5 followed by replacement of zinc with a given metal.

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/min/μmole of enzyme, where a_0 and a represent the concentration of substrate at time 0 and time t, respectively (Riordan and Vallee, 1963). The assays were carried out at 0° in 0.02 M sodium Veronal–1.0 M NaCl buffer, pH 7.5. *C* was calculated from the linear portion of the first-order reaction plots before hydrolysis exceeded 15%. *C* values for peptidase activity of the native zinc enzyme ranged from 30 to 36, while those of the acetylated and iodinated zinc enzymes ranged from 0 to 0.7.

Esterase activity was determined by 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 constant, k, with units of milliequivalents of H^+ per minute per micromole of enzyme; k values varied from 6 to 7.5 × 10^3 for the native enzyme, from 38 to 45 × 10^3 for the acetylated, and from 25 to 33 × 10^3 for the iodinated zinc 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 δ- and γ-carboxypeptidase A (Bargetzi *et al.*, 1963) is 6.42 × 10^4 $mole^{-1}$ (Simpson *et al.*, 1963).

[1] Abbreviations: The abbreviations used are in the formulations and figures only and when required for differentiation: [(CPD)Zn], zinc carboxypeptidase; [Ac_I(CPD)Zn], acetyl zinc carboxypeptidase, acetylated with *N*-acetylimidazole; [Iodo-(CPD)Zn], iodinated zinc carboxypeptidase. [CPD] represents the apoenzyme, and the brackets indicate the firm binding of zinc or other metals substituting for it, *e.g.* [(CPD)Hg.] Carboxypeptidase refers to carboxypeptidase A only; CGP, carbobenzoxyglycyl-L-phenylalanine; HPLA, hippuryl-*dl*-β-phenyllactate.

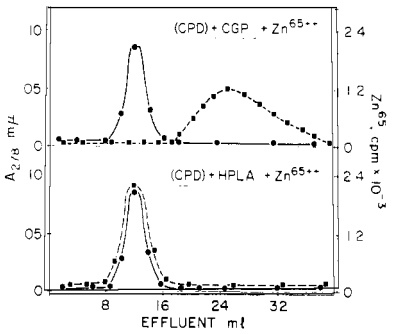

FIGURE 1: Prevention of $^{65}Zn^{2+}$ binding to apocarboxypeptidase (CPD) by a peptide substrate, carbobenzoxyglycyl-L-phenylalanine (CGP), and binding of $^{65}Zn^{2+}$ to apocarboxypeptidase in the presence of an ester substrate, hippuryl-*dl*-β-phenyllactate (HPLA). Peptide: (CPD), 5×10^{-6} M, plus CGP, 4×10^{-2} M, were preincubated for 2 min, then $^{65}Zn^{2+}$ ions, 5×10^{-6} M, were added. All components were in 1 M NaCl–0.05 M Tris, pH 7.5, 4°. After mixing for 1 min, a 1-ml sample was passed over a 1 × 30 cm, G-25 Sephadex column at a flow rate of 1 ml/min. Absorbance at 278 mμ (●) and $^{65}Zn^{2+}$ (■), counts per minute per successive 2-ml fractions were determined. Ester: the procedure was identical with that described above except for the substitution of 4×10^{-2} M *dl*-HPLA as the substrate.

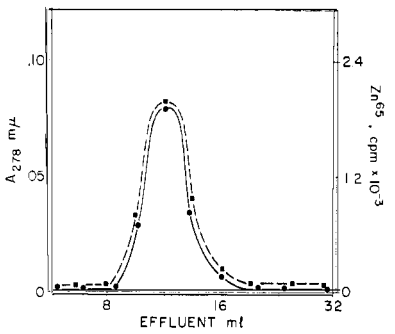

FIGURE 2: Binding of $^{65}Zn^{2+}$ to acetyl- and iodoapocarboxypeptidase, (Ac_1CPD) and (IodoCPD), in the presence of peptide substrate, carbobenzoxyglycyl-*l*-phenylalanine (CGP), and ester substrate, hippuryl-*dl*-β-phenyllactate (HPLA). Peptide: samples of acetyl- and iodocarboxypeptidase, 5×10^{-6} M, plus CPG, 4×10^{-2} M, were preincubated for 2 min, then $^{65}Zn^{2+}$ ions, 5×10^{-6} M, were added. All components were in 1 M NaCl–0.02 M sodium Veronal, pH 7.5, 4°. After mixing for 1 min, a 1-ml sample was passed over a 1 × 30 cm, G-25 Sephadex column at a flow rate of 1 ml/min. Absorbance at 278 mμ (●) and $^{65}Zn^{2+}$ (■), counts per minute per successive 2-ml fractions were determined. Ester: the procedure was identical with that described above except for the substitution of 4×10^{-2} M *dl*-HPLA as the substrate. The results were superimposable on those obtained with carbobenzoxyglycyl-*l*-phenylalanine (CGP).

Gel Filtration and Metal Ion Exchange. The gel filtration method utilizing $^{65}Zn^{2+}$ for the detection of apocarboxypeptidase–substrate complexes (Coleman and Vallee, 1962a) and methods for the detection of metallocarboxypeptidase–substrate complexes based on metal ion exchange have been described (Coleman and Vallee, 1962b) and are illustrated by Figure 1 for ready comparison of the previous data with those to be reported here.

Results

Under optimal conditions (Wacker *et al.*, 1964; Riordan *et al.*, 1965b), both acetylation and iodination virtually abolish the ability of carboxypeptidase to hydrolyze CGP (Vallee *et al.*, 1963; Riordan and Vallee, 1963; Simpson *et al.*, 1963; Simpson and Vallee, 1966; R. C. Davies, J. F. Riordan, and B. L. Vallee, in preparation). Under the conditions of the experiments, this substrate prevents the binding of $^{65}Zn^{2+}$ to apocarboxypeptidase (Figure 1), but not to acetyl- or iodoapocarboxypeptidases (Figure 2). The reconstituted, ^{65}zinc-labeled acetyl enzyme exhibits 700% of the esterase but virtually none of the peptidase activity of the native enzyme. Like carbobenzoxyglycyl-L-phenylalanine, glycyl-L-phenylalanine, glycyl-L-tyrosine, and glycyl-L-leucine fail to affect $^{65}Zn^{2+}$ binding to acetyl- or iodocarboxypeptidase. After the tyrosyl residues of acetylcarboxypeptidase were deacylated with hydroxylamine (Riordan and Vallee, 1963) and zinc was removed, the resultant apocarboxypeptidase was reconstituted by the addition of $^{65}Zn^{2+}$. Subsequent to deacylation, the esterase and peptidase activities were indistinguishable from those of the native enzyme. As would be expected, carbobenzoxyglycyl-L-phenylalanine now again prevents binding of $^{65}Zn^{2+}$ to the apoenzyme resulting in a Sephadex elution pattern identical with that in Figure 1.

Copper, cadmium, or mercury carboxypeptidases do not hydrolyze peptides; hence, the effect of peptide substrates on the exchange of these ions can be studied readily. In a typical control experiment (Figure 3), $^{65}Zn^{2+}$ exchanges freely and rapidly with the copper of [(CPD)Cu], and substrates like glycyl-L-phenylalanine, glycyl-L-tyrosine, glycine-L-leucine, or carbobenzoxyglycyl-L-phenylalanine, 2×10^{-2} M, prevent the exchange; but the same concentrations of these substrates do not prevent analogous exchanges of metals, either with acetyl- or iodocarboxypeptidases (Figure 4).

Thus, both the gel filtration and isotope methods indicate that such modifications interfere with the binding of peptide substrates, and these physicochemical results are entirely consistent with the observed decrease of peptidase activity. These studies offer little information regarding the basis of the substantial increase in esterase activities at these substrate concentrations observed for many derivatives studied thus far (Table I). The substitution of Mn^{2+}, Co^{2+}, and Ni^{2+} for Zn^{2+} does not alter esterase activity of the

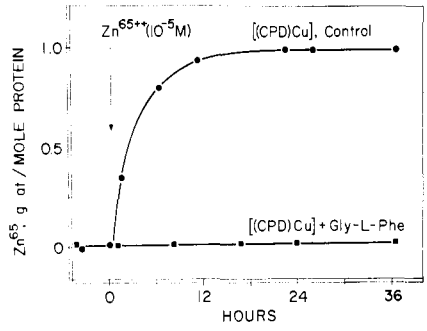

FIGURE 3: Exchange of $^{65}Zn^{2+}$ for copper in copper carboxypeptidase in the presence and absence (control) of peptide substrate, Gly-L-Phe. As a control, 1×10^{-5} M copper carboxypeptidase was exposed to 1×10^{-5} M $^{65}Zn^{2+}$ at 0 time and the $^{65}Zn^{2+}$ bound by the enzyme (●) measured as dialysis progressed. Copper carboxypeptidase, 1×10^{-5} M, was then equilibrated with 4×10^{-2} M Gly-L-Phe for 2 hr, followed by the addition of 1×10^{-5} M $^{65}Zn^{2+}$ at 0 time. $^{65}Zn^{2+}$ bound by the enzyme (■) was measured as dialysis progressed. Radioactivity bound to the enzyme was measured as previously described (Coleman and Vallee, 1962b).

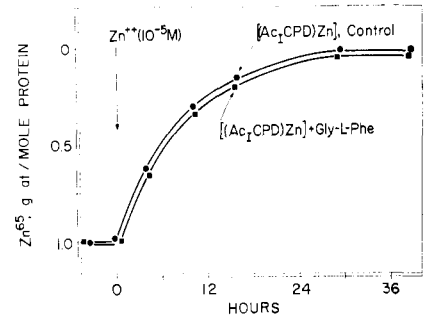

FIGURE 4: Exchange of Zn^{2+} for ^{65}Zn in acetylcarboxypeptidase labeled with ^{65}Zn in the presence and absence (control) of peptide substrate, Gly-L-Phe. As a control, 1×10^{-5} M [(Ac_1CPD)^{65}Zn] was exposed to 1×10^{-5} M Zn^{2+} at 0 time and the $^{65}Zn^{2+}$ bound by the enzyme (●) measured as dialysis progressed. Acetylcarboxypeptidase labeled with ^{65}Zn, 1×10^{-5} M, was then equilibrated with 4×10^{-2} M Gly-L-Phe for 2 hr, followed by the addition of 1×10^{-5} M Zn^{2+} at 0 time. ^{65}Zn bound to the enzyme (■) was measured as dialysis progressed.

native enzyme markedly, but Cd^{2+} and Hg^{2+} increase it and Cu^{2+} abolishes it (Table I).

The esterase activity of cadmium carboxypeptidase, initially higher than that of the native zinc enzyme, remains virtually the same on acetylation or iodination. Mercury carboxypeptidase, an active esterase, is rendered inactive either by acetylation or iodination. Copper carboxypeptidase, inactive to begin with, remains inactive on acetylation or iodination (Table I).[2]

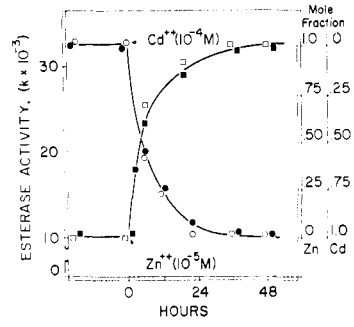

FIGURE 5: Esterase activities and metal contents during the replacement of ^{115m}Cd in [(Ac_1CPD)^{115m}Cd] by Zn^{2+} and during the replacement of ^{65}Zn in [(Ac_1CPD)^{65}Zn] by Cd^{2+}. The ^{115m}Cd and ^{65}Zn acetyl-enzymes, 1×10^{-5} M, were dialyzed *vs.* the concentrations of Zn^{2+} and Cd^{2+} indicated in the figure, contained in 1 M NaCl–0.02 M Veronal, pH 8.0, 4°. Radioactivity bound to the protein was determined as previously described (Coleman and Vallee, 1960) and esterase activity determined as described under methods. (■), mole fraction of ^{115m}Cd contained in [(Ac_1CPD)^{115m}Cd] during zinc exchange. (●), mole fraction of ^{65}Zn contained in [(Ac_1CPD)^{65}Zn] during cadmium exchange. (■), esterase activity of [(Ac_1CPD)^{115m}Cd] during zinc exchange. (●), esterase activity of [(Ac_1CPD)^{65}Zn] during cadmium exchange.

During the time course of acetylation with *N*-acetylimidazole, the esterase activities of zinc, manganese, cobalt, and nickel carboxypeptidases increase rapidly on exposure to *N*-acetylimidazole to become distinctive and constant after *ca.* 60 min. The marked differences in the esterase activities of cadmium and mercury carboxypeptidases in response to acetylation or iodination (Table I) compared to those of the metalloenzymes formed from the apoenzyme and the first transition metals suggested either that these group IIB metal ions interact with ligand sites on the enzyme surface different from those binding zinc and the transition metals, or that the chemical modifications induce a change which in the presence of Hg^{2+} or Cd^{2+} result in substrate binding to different residues of the enzyme. Such conjectures are encouraged by the activities observed on equilibrium dialysis of Zn^{2+} *vs.* the cadmium or mercury acetyl- or iodocarboxypeptidases, enzymatically inactive toward peptides (Figures 5 and 6). Replacement of these metals by zinc completely restores the activities to those which have been found to be characteristic of the respective zinc enzymes. Both the amounts of zinc, cadmium, or mercury bound to the enzyme and the esterase activities were measured simultaneously at various times during the exchange. The results demonstrate that the changes in activities are a

[2] Earlier data (Coleman *et al.*, 1964) indicating that iodination or acetylation induce esterase activity in copper carboxypeptidase have proven to be due to zinc contamination of one of the reagents employed.

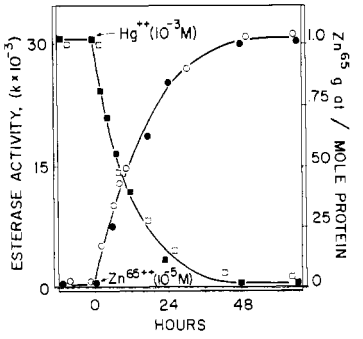

FIGURE 6: Esterase activities and enzyme-bound ^{65}Zn during the replacement of mercury in [(IodoCPD)Hg] by $^{65}Zn^{2+}$ and during the replacement of ^{65}Zn in [(IodoCPD)^{65}Zn] by mercury. The mercury and ^{65}Zn iodocarboxypeptidases were dialyzed *vs.* the concentrations of $^{65}Zn^{2+}$ and Hg^{2+} indicated in the figure, contained in 1 M NaCl–0.05 M Tris, pH 8.0, 4°. (●), mole fraction of ^{65}Zn bound to enzyme during replacement of mercury by $^{65}Zn^{2+}$. (■), mole fraction of ^{65}Zn bound to the enzyme during the replacement of ^{65}Zn by Hg^{2+}. (●), esterase activity during the replacement of mercury by $^{65}Zn^{2+}$. (■), esterase activity during the replacement of ^{65}Zn by Hg^{2+}. Radioactivity and esterase activities were measured as in Figure 5.

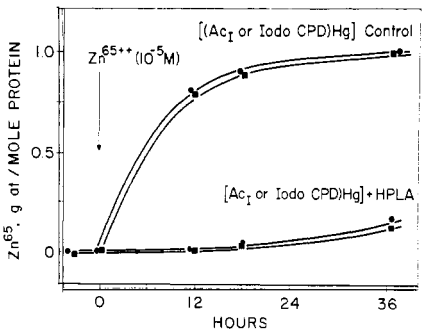

FIGURE 7. Exchange of $^{65}Zn^{2+}$ for mercury in acetyl- and iodomercurycarboxypeptidases, [(AcıCPD)Hg] and [(IodoCPD)Hg], in the presence and absence (controls) of the ester substrate (HPLA). As controls, [(Ac$_1$CPD)-Hg] (●) and [(IodoCPD)Hg] (■), both 1×10^{-5} M, were dialyzed *vs.* $^{65}Zn^{2+}$, 1×10^{-5} M, beginning at 0 time, and enzyme-bound ^{65}Zn (●, ■) measured as a function of time. Samples of [(Ac$_1$CPD)Hg] (●) and [(IodoCPD)Hg] (■), 1×10^{-5} M, were equilibrated *vs.* 4×10^{-2} M HPLA for 2 hr, $^{65}Zn^{2+}$, 1×10^{-5} M, was then added at 0 time, and enzyme-bound ^{65}Zn (●, ■, lower curve) followed as a function of time.

TABLE I: Activities of Chemically Modified Metallocarboxypeptidases, [(CPD)Me].[a]

Me	Native C	Native $k \times 10^{-3}$	Acetyl[b] $k \times 10^{-3}$	Iodo[b] $k \times 10^{-3}$
Zn	34	7	45	33
Mn	9.5	11[c]	27[c]	33
		(3)	(8)	
Co	73	8	46	32
Ni	17	3	19	20
Cd	0	10	11	11
Hg	0	6	0	0
Cu	0	0	0	0

[a] Prepared from carboxypeptidase A (Allan *et al.*, 1964). Peptidase assays were performed at 0°, esterase assays at 25°. [b] Acetyl- and iodometallocarboxypeptidases exhibited either negligible or no peptidase activity (see text). [c] Maximum activity of manganese carboxypeptidases requires assay in the presence of excess metal; the values in parentheses are those obtained upon the addition of only a 1:1 molar ratio.

direct function of the amounts of each of the two exchanging species present at the active site at any one time.

Exposure of [(Ac$_1$CPD)^{65}Zn] to excess Cd^{2+} ions lowers the esterase activity from 36, typical for this zinc enzyme, to 10, characteristic of [(Ac$_1$CPD)Cd], and, simultaneously, cadmium completely displaces ^{65}Zn from the enzyme. Similarly, exposure of [(AcCPD)^{115m}Cd] to an excess of Zn^{2+} ions increases the esterase activity from 10 to 36 while zinc concurrently displaces the ^{115m}Cd bound to the enzyme. Chemical measurements of cadmium and zinc confirmed their mutual replacements (Figure 5).

The experiments with [(IodoCPD)Hg] were performed in similar fashion. On substituting Hg^{2+} for the zinc of [(IodoCPD)Zn], esterase activity is lost completely. It is then restored by displacing mercury with $^{65}Zn^{2+}$ (Figure 6). At the end of this experiment the enzyme contains 1 g-atom of ^{65}Zn, but no mercury.

When a derivative of carboxypeptidase is an active esterase the binding of the ester cannot be examined readily by means of the metal ion exchange procedure. Nor can gel filtration, of course, reveal changes of ester binding to the native, the acetyl, or the iodo enzymes (Figures 1 and 2) (Coleman and Vallee, 1962b). Since the acetyl or iodo mercury carboxypeptidases are inactive as esterases, it seemed important to establish whether or not the loss of esterase activities can be attributed to a complete loss of the capacity of these enzymes to bind the ester substrate.

The mercury of both acetyl- and iodocarboxypeptidase exchanges rapidly for $^{65}Zn^{2+}$, but hippuryl-*dl*-β-phenyllactate *prevents* this exchange (Figure 7). This indicates that hippurylphenyllactate can form a complex with both the acetyl and iodo mercury enzymes, but in neither case does this lead to hydrolysis.

Discussion

Previous work has elucidated a number of the chemical features of the active center of carboxypeptidase. A series of metal ion substitutions and chemical modifications of the metal-binding groups have demonstrated the features of the metal-binding site. The species of metal ion at the active site determines both the magnitude of the specific activity and the specificity of the enzyme (Coleman and Vallee, 1961). The protein-binding groups of the substrate have been defined by kinetic studies (Smith, 1951; Smith and Lumry, 1950; Neurath and Schwert, 1950) and through the effect of substrates on metal ion binding and exchange (Coleman and Vallee, 1962a,b). To the extent that these methods permit a decision the metal ion is apparently indispensable for the binding of esters, though peptides will still bind to the apoenzyme after the metal has been removed.

Chemical modifications of carboxypeptidase have identified two tyrosyl residues which are critical to function. Acetylation or iodination of the tyrosyl residues markedly decreases peptidase activity and increases esterase activity five- to sevenfold (Simpson *et al.*, 1963; Riordan and Vallee, 1963; Simpson and Vallee, 1966). Similar data have been obtained for acetylcarboxypeptidase by Bender and co-workers (Bender *et al.*, 1965; Whitaker *et al.*, 1966).

In the present work we have employed a combination of the previous approaches in order to define better the role of the tyrosyl residues which might participate in substrate binding or hydrolysis or both, and to examine the possible relationships between various metal ions and the organic groups which may be involved in the catalytic process. While such interrelationships are usually examined by kinetic measurements the system under study permits the application of various equilibrium procedures to obtain pertinent information. In this manner, a selection of suitable systems for detailed kinetic analysis might be feasible from the vast number of alternatives now at hand, and these present studies were undertaken in large measure to permit such choices.

The gel filtration and isotope exchange experiments demonstrate that acetylation or iodination of two tyrosyl residues (Simpson *et al.*, 1963; Riordan and Vallee, 1963; Simpson and Vallee, 1966) interferes with the binding of the peptide substrate to the active center, thereby accounting for the loss of peptidase activity (Figures 2 and 4) (Coleman *et al.*, 1964; Vallee, 1964a). The methods here employed are incapable, of course, of determining whether or not the blockage of these residues completely abolishes the binding of peptide substrates, since the effect of the substrates on metal binding or exchange are indirect gauges. Further studies with different concentrations, isotopically labeled substrates, and variations of the physicochemical conditions are required to yield quantitative information concerning the *degree* to which binding is blocked.

Nor can these studies eliminate the possibility that chemical modification of one or several groups involved in the catalytic step might contribute to the loss of peptidase activity. Thus, one or both of the active center tyrosyl residues may be involved in peptide binding. It has not proven possible thus far to prepare an enzyme in which only one of these two residues is acetylated, and the possibility that one of the two tyrosyl residues participates in the catalytic mechanism cannot be excluded on the basis of current data or the observed substrate inhibition.

Peptidase activity is virtually abolished when chemical modifications are performed under optimal conditions. In that instance the ninhydrin values are indistinguishable from those of the control in the absence of enzyme, and there is no increase in color during the period of assay. We have attributed the low peptidase activity observed in some instances (Riordan and Vallee, 1963; Bender *et al.*, 1965) to the presence of a small fraction of molecules whose active centers are unmodified (Bethune *et al.*, 1964). This interpretation is also consistent with the observation that deacetylation of the acetyl enzyme with hydroxylamine jointly restores both peptide binding and peptidase activity to levels observed in the native enzyme. Hence, the loss of peptidase activity and peptide binding on acetylation cannot be attributed to an irreversible change in protein secondary or tertiary structure. Though the consequences of iodination of the enzyme have not proven reversible thus far, the similarity of the binding studies and of the enzymatic changes to those observed on acetylation (Vallee *et al.*, 1963) are consistent with the assumption that iodination, like acetylation, affects peptidase activity by interfering with peptide binding (Simpson and Vallee, 1966).

The C-terminal amino acids of the most effective dipeptide substrates of carboxypeptidase contain aromatic or large aliphatic substituents. Hence, they could form hydrophobic bonds, in this instance with one or both of the tyrosyl residues, thereby affecting the relative rates of hydrolysis of substrates containing different R groups.

Since both the acetyl- and iodocarboxypeptidase remain active esterases, both of the tyrosyl residues are apparently not required to bind the esters (Table I). Only a very limited number of ester substrates of carboxypeptidase have been prepared and, hence, it cannot be stated at this juncture to what extent the R group of the C-terminal residue is critical for their hydrolysis.

The enzymatic consequences of acetylation and iodination of tyrosyl residues are qualitatively similar to those observed on substitution of cadmium or mercury for zinc at the active site of the enzyme; under comparable conditions, both result in an increased esterase and loss of peptidase activity (Vallee *et al.*, 1963). But in contrast to acetyl- and iodocarboxypeptidases the cadmium or mercury enzymes like the unmodified copper enzyme bind peptide substrates (Figure 3). Thus, even though the enzymatic end result is similar, the manner in which it is brought about obviously differs (Coleman *et al.*, 1964; Vallee, 1964b). It may be presumed that cadmium or mercury carboxypeptidases fail to initiate peptide hydrolysis of benzoyl-

glycyl-L-phenylalanine though they can hydrolyze hippuryl-*dl*-β-phenyllactic acid (Vallee, 1961). Since structurally these substrates differ only in the peptide *vs.* the ester bond, it would be difficult to hold steric factors alone accountable for the altered substrate specificity.

The present data offer an explanation for the curtailment of the dual specificity, but do not indicate the mechanism which increases the esterase activities of the acetyliodo- and metal-substituted enzymes compared to those of native carboxypeptidase. This could be achieved through changes in K_m, V_{max}, substrate inhibition, or their combination (Simpson *et al.*, 1963; Riordan and Vallee, 1963; McClure *et al.*, 1964). Kinetic studies (Riordan *et al.*, 1965a), and others now in progress, in conjunction with previous work (Riordan and Vallee, 1963) should clarify these problems.

As has been noted, cobalt, nickel, and manganese, metals of the first transition series, do not change esterase activity significantly (Table I). Acetylation and iodination of these metalloenzymes practically abolishes peptidase activity.

However, these same organic modifications variously and differently affect the respective esterase activities (Table I). In each instance, the final activity achieved appears to be governed by the particular metal ion, though, of themselves, the respective substitutions in the native enzyme do not result in significantly increased esterase activities (Table I). Since the assays are performed in a range of substrate concentration which results in substrate inhibition, a resolution of these problems will have to be deferred until the kinetics of these enzymes are explored more completely.

Copper carboxypeptidase is the least stable of the metallocarboxypeptidases and hence most difficult to prepare in a zinc-free form. By repeated purification of all reagents employed, iodo–acetyl copper carboxypeptidases could be demonstrated to be inactive toward esters (Table I). Therefore, neither copper carboxypeptidase nor acetyl or iodo copper carboxypeptidases can hydrolyze either peptides or esters, perhaps due to the geometry of copper complexes (Cotton and Wilkinson, 1962).

The consequences of acetylation and iodination on the esterase activities of the cadmium and mercury carboxypeptidases, both higher than that of the native enzyme, are perhaps the most revealing in this entire series of modifications. Under the conditions of assay employed for the native enzymes, acetylation or iodination virtually does not alter the observed esterase activity of cadmium carboxypeptidase. The reasons for this have been explored (Riordan *et al.*, 1965a) and will be documented further (R. C. Davies, J. F. Riordan, and B. L. Vallee, in preparation). These modifications completely abolish the esterase activity of mercury carboxypeptidase.

In order to eliminate the possibility that mercury or cadmium protects tyrosyl or other residues against iodination or acetylation, these ions were exchanged with zinc *subsequent* to the modification reactions. The zinc enzyme formed in this manner exhibits characteristics identical with those observed when this metal has been present in the enzymes throughout the entire procedure (Figures 5 and 6). Clearly, the cadmium and mercury enzymes are fully iodinated and acetylated, and the enzymatic properties cannot be attributed easily to the protection of functional groups. Further, since the activity can be fully restored to that of the zinc enzyme, irreversible changes in protein secondary or tertiary structure cannot be held accountable readily for the loss of esterase activity. Nor can it be attributed to the blocking of a group involved in ester substrate binding since these enzymes still bind esters (Figure 7). The reasons for the loss of peptidase activity on substituting cadmium and mercury for zinc and the loss of esterase activity on acetylation or iodination of mercury carboxypeptidase are continuing subjects of study. In this regard the geometry of mercury complexes which differ substantially from those of the other IIB metals is the focus of particular attention.

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Some Catalytic Properties of Human Liver Alcohol Dehydrogenase*

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ABSTRACT: Human liver alcohol dehydrogenase purified by means of DEAE- and CM-cellulose chromatography can be resolved into three chromatographically distinct, enzymatically active forms. One of these was studied in detail and found to exhibit broad substrate specificity characteristic of the unresolved material. In addition to ethanol, methanol, 1,2-ethanediol, 1,2-propanediol, 1,3-propanediol, 2-methoxyethanol, and 2-ethoxyethanol are oxidized. The oxidation of 1,2-propanediol is competitive with that of ethanol. The enzyme also oxidizes monohalo, but not di- or trihalo derivatives of ethanol, and reduces monochloro, dichloro, and trihalo derivatives of acetaldehyde. Both the polyhalo derivatives of ethanol and of acetaldehyde inhibit ethanol oxidation. These enzymatic findings are pertinent to the metabolism of these alcohols and aldehydes in man.

Alcohol dehydrogenase from human liver has been purified substantially (von Wartburg *et al.*, 1964). Zinc is a functional constituent of the molecule, and like other zinc metalloenzymes, the activity of alcohol dehydrogenase is inhibited in a characteristic manner by chelating agents such as 1,10-phenanthroline. Many of the catalytic properties of the human protein are similar to those of the enzyme from horse liver, but they differ in regard to the magnitudes of the Michaelis constants for substrates and coenzymes and in substrate specificity (von Wartburg *et al.*, 1964). In particular, human liver alcohol dehydrogenase oxidizes ethylene glycol and methanol at significant rates whereas the horse enzyme exhibits little or no activity toward these alcohols (Sund and Theorell, 1963; Winer, 1958; Theorell and Bonnichsen, 1951). These observations are of importance since the metabolic products of ethylene glycol and methanol are toxic to man and the enzyme may play a role in adverse reactions to these and other compounds (Wacker *et al.*, 1965). This paper reports further studies on the purification of human liver alcohol dehydrogenase and on its behavior toward other aliphatic diols and a number of halogenated derivatives of ethanol and acetaldehyde.

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