Carboxypeptidase A. Differences in the Mechanisms of Ester and Peptide Hydrolysis†

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ABSTRACT: A series of depsipeptides whose structures complement exactly those of oligopeptide substrates studied previously has been examined to explore the dual specificity of carboxypeptidase A. They have the general form R-(Gly)$_n$-L-OPhe where OPhe is phenyllactate and R is benzoyl, benzyloxycarbonyl, or 5-dimethylaminonaphthalene-1-sulfonyl and n = 2 or 3. Like that of their oligopeptide analogs the hydrolysis of these esters conforms to Michaelis–Menten kinetics. Several lines of evidence indicate that the mechanism of hydrolysis of these esters differs from that of their exact peptide analogs. Direct examination of enzyme–substrate complexes demonstrates that the mode of binding of esters to the active center differs significantly from that of their analogous peptides. Thus, noncompetitive inhibitors of peptide hydrolysis are competitive inhibitors of ester hydrolysis. The apoenzyme binds peptides as tightly as the zinc enzyme but esters much more weakly. Metal substitution at the active site profoundly affects the rate determining step in the hydrolysis of peptides (k_{cat} values are in the order: Co > Zn > Mn > Cd) but not their binding. Conversely, metal substitution affects binding of the esters (K_m^{-1} values in the order: Co > Zn > Mn > Cd) but not their rate of hydrolysis. These results would be expected if the metal atom were to interact with the carboxyl group of esters during the binding step but with the carbonyl group of peptides during the catalytic step. The present studies raise questions as to the mechanistic importance of a substrate-induced conformational change of Tyr-248 initiated by interaction of the substrate carboxyl group with Arg-145.

The results of numerous studies of carboxypeptidase A have generated considerable discussion as to whether or not this enzyme hydrolyzes esters and peptides by the same mechanism (Vallee *et al.*, 1963; Kaiser and Carson, 1965; Whitaker *et al.*, 1966; Lipscomb *et al.*, 1968). Its dual specificity can be altered either by replacement of the native zinc ion at the active site with other metals or by chemical modification of its amino acid side chains (Vallee *et al.*, 1963, 1970). In many of these instances peptidase activity toward Cbz-Gly-Phe decreases while esterase activity toward Bz-Gly-OPhe[1] actually increases. This dipeptide and its ester analog have traditionally been employed in the conventional assay procedures for carboxypeptidase. However, kinetic anomalies associated with these substrates have severely hindered discernment of the mechanisms underlying their differential responses to enzyme modification. On the other hand the hydrolyses of Cbz-Gly-Gly-Phe and other N-blocked oligopeptides are devoid of such anomalies (Auld and Vallee, 1970a). Hence they are suitable for mechanistic studies (Auld and Vallee, 1970a,b, 1971; Auld *et al.*, 1972).

In the present investigation, a series of depsipeptides, whose structures complement exactly those of oligopeptide substrates studied previously, is examined to explore the dual specificity of carboxypeptidase. Importantly the hydrolysis of these esters also conforms to Michaelis–Menten kinetics. Moreover the use of fluorescent N-terminal blocking groups allows direct observation of their enzyme·substrate complexes (Latt *et al.*, 1970, 1972; Auld *et al.*, 1972). These matched peptide–ester pairs, therefore, provide a unique opportunity to study in depth the nature of ester and peptide hydrolysis catalyzed by carboxypeptidase. The results demonstrate unequivocally that the mechanism of hydrolysis of peptides and esters differs. Preliminary communications of this work have been published (Auld and Holmquist, 1972, 1973).

Materials and Methods

The depsipeptide and oligopeptide substrate pairs studied have the general form R-(Gly)$_n$-X-CH(R′)COOH where X = O or NH. Each ester–peptide pair differs only at the susceptible bond. For the peptide, the C-terminal residue is an L-amino acid, while for the ester it is the corresponding β-substituted L-lactic acid. The exact ester analog for the peptide substrate containing the phenylalanyl residue (X = NH, R′ = CH_2Ph) is therefore the L-phenyllactate derivative (X = O, R′ = CH_2Ph). The N-terminal blocking group, R, is either benzoyl, carbobenzoxy, or the fluorescent dansyl group. The length of the substrate is varied by the number, n, of glycyl residues. The synthesis of oligopeptides has been described (Auld and Vallee, 1970a; Latt *et al.*, 1972). The ester substrates were synthesized in a manner similar to the procedures of Gisin *et al.* (1969) and details will be given elsewhere (B. Holmquist and D. S. Auld, manuscript in preparation).

Bovine pancreatic carboxypeptidase A prepared according to the procedure of Anson (1937) or Cox *et al.* (1964) was obtained from Worthington Biochemical Corporation

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[1] Abbreviations used are: OPhe, α-hydroxy-β-phenyllactate; OLeu, α-hydroxy-β-isopropyllactate; Dns, 5-dimethylaminonaphthaline-1-sulfonyl (dansyl); Mes, 2-(N-morpholino)ethanesulfonic acid. The term depsipeptide is defined here as an oligopeptide which has an ester bond only at the site of carboxypeptidase cleavage. The ester Bz-Gly-OPhe has been referred to in earlier work as HPLA. [(CPD)Me] refers to metallocarboxypeptidase A where (CPD) represents the apoenzyme and the brackets indicate the firm binding of metal, Me, where Me is Zn, Cd, Co, or Mn.

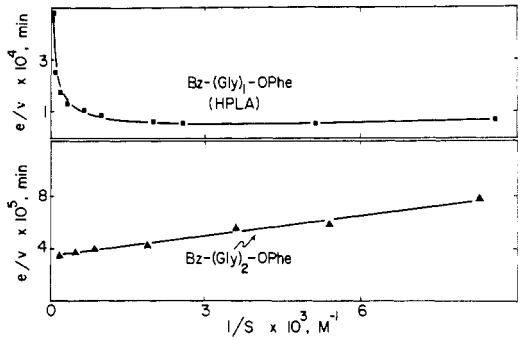

FIGURE 1: Lineweaver-Burk plots for carboxypeptidase A (Cox) catalyzed hydrolysis of Bz-Gly-L-OPhe (HPLA) and Bz-(Gly)$_2$-L-OPhe, measured at 25° and pH 7.5 in 10^{-4} M Tris–1.0 M NaCl.

or Sigma Chemical Corporation, respectively.[2] The corresponding apoenzymes were prepared as follows. Enzyme crystals were suspended (5 mg/ml) in 10 mM 1,10-phenanthroline, 1 mM Mes[1] (pH 7.0) at 25° for 1 hr for four successive times followed by at least four 0.5-hr washings with 1 mM Mes (pH 7.0). The crystalline apoenzyme was reconstituted by the addition of a threefold excess of the desired metal, followed by three washings with 1 mM Mes (pH 7.0). The apoenzymes and metallocarboxypeptidases, [(CPD)Me], prepared in this manner contained less than 0.003 g-atom of zinc/mol of enzyme, as determined by atomic absorption spectroscopy (Fuwa *et al.*, 1964). All assays of [(CPD)Me] other than zinc were performed in the presence of 10^{-4} M metal ion.

The preparation and storage of enzyme solutions, precautions taken for metal-free conditions, and the procedures used for peptidase assays have been detailed (Auld and Vallee, 1970a,b). Esterase assays were performed by titration of the protons released on hydrolysis using 2 mM NaOH and a Radiometer titrator comprised of an ABU 12 autoburette coupled to a TTT 11 autotitrator, TTA 31 titration assembly, and a PHM 28 pH meter. All rates were measured at 25 ± 0.1°. Stopped-flow fluorescence measurements utilized a Durrum-Gibson instrument equipped with the Durrum fluorescence accessory No. 16400, a 75-W xenon lamp, and an end-on EMI 9526B photomultiplier. Details of this technique have also been described previously (Latt *et al.*, 1972).

Results

Bz-Gly-L-OPhe, which has long served to measure esterase activity of carboxypeptidase (Snoke and Neurath, 1949), exhibits marked substrate inhibition, as evidenced by the upward curvature of the Lineweaver-Burk plot at substrate concentrations greater than 1 mM (Figure 1, top) (McClure *et al.*, 1964). However, for the diglycyl substrate, Bz-Gly-Gly-L-OPhe, the Lineweaver-Burk plot is linear over a wide concentration range (Figure 1, bottom). Michaelis-Menten kinetics are also found for the benzoyl, carbobenzoxy, and dansyl blocked depsipeptides where $n = 3$. The substrate activation observed in the hydrolysis of dipeptide substrates also disappears when tripeptide or higher homologs are examined (Auld and Vallee, 1970a). The ability to determine the kinetic parameters k_{cat} and K_m for these longer substrates allows delineation of the source of various differences in ester and peptide hydrolysis.

TABLE I: Hydrolysis of Matched Ester and Peptide Pairs by [(CPD)Zn] and [(CPD)Cd].[a]

	[(CPD)Zn]		[(CPD)Cd]	
Substrate	k_{cat} (min^{-1})	$10^4 K_m$ (M)	k_{cat} (min^{-1})	$10^4 K_m$ (M)
Bz-(Gly)$_2$-L-OPhe	30,000	3.3	34,000	79.0
Bz-(Gly)$_3$-L-OPhe	31,000	3.4	45,000	29.0
Dns-(Gly)$_3$-L-OPhe	11,000	0.25	28,000	2.2
Bz-(Gly)$_2$-L-Phe	1,200	10.0	41	8.0
Bz-(Gly)$_3$-L-Phe	2,600	37.0	86	41.0
Dns-(Gly)$_3$-L-Phe	4,200	8.0	400	8.1

[a] Assays performed at 25°, pH 7.5, 1.0 M NaCl, and a buffer concentration of 0.05 M Tris for peptide hydrolysis and 10^{-4} M Tris for ester hydrolysis. The Anson enzyme was used in all cases.

Inorganic Chemical Modification. The replacement of the zinc of carboxypeptidase with cadmium results in one such difference. Activity toward dipeptides markedly decreases while increasing slightly toward the ester, Bz-Gly-L-OPhe (Coleman and Vallee, 1960). At identical substrate concentrations, the activity of the Cd enzyme toward the di- and triglycyl homologs of this ester also increases. A comparison of the kinetic parameters for substrate hydrolysis by the Zn and Cd enzymes clearly establishes the origin of this differential response (Table I). The increased esterase activity of the Cd enzyme is reflected in a slightly higher k_{cat} value. However, the corresponding K_m values are 10- to 20-fold higher than those for the Zn enzyme. The exact opposite relationship is found for their matched peptide analogs. The decreased activity of the Cd enzyme toward peptides is due to drastically reduced k_{cat} values, *i.e.* 10- to 30-fold. K_m values remain constant, however.

Both steady-state kinetic and stopped-flow fluorescence studies have shown previously that K_m values for peptide hydrolysis catalyzed by the native enzyme are equivalent to dissociation constants, K_D, of the Michaelis complex (Auld and Vallee, 1970a; Auld *et al.*, 1972). The nearly identical K_m values obtained for the Zn and Cd enzymes acting on the peptide substrates therefore suggest that these enzymes bind peptides to nearly the same extent. Stopped-flow fluorescent assays of the *N*-dansylated peptide substrate, Dns-(Gly)$_3$-L-Phe, served to verify this conclusion (Figure 2). For both enzymes, rapid binding takes place during the mixing time of the instrument, as indicated by the rapid decrease in enzyme tryptophan fluorescence upon mixing enzyme and substrate. For the Zn enzyme·substrate complex, the signal returns to the base line in a few tenths of a second as hydrolysis is completed. For the Cd enzyme·substrate complex the much slower return of the signal to the base line reflects a much slower rate of hydrolysis. Hence, the reduced activity of the Cd enzyme toward peptides is clearly due to reduced catalytic efficiency, not to lack of peptide binding.

Substitution of cobalt or manganese for zinc also affects peptide and ester hydrolysis (Table II). The k_{cat} values for Bz-(Gly)$_2$-L-Phe hydrolysis follow the order Co > Zn >

[2] Kinetic parameters obtained for the Cox and Anson enzymes are nearly identical and therefore both enzymes have been used in this study. Measurements of substrate binding to the apoenzyme necessitated the use of the Cox enzyme because of its greater stability in solution.

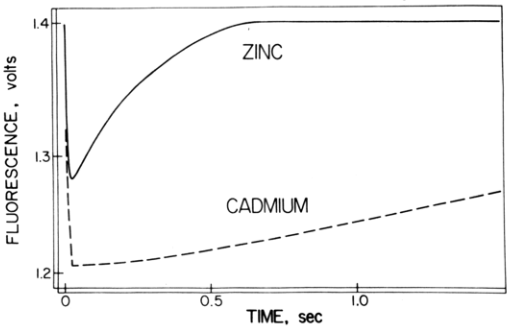

FIGURE 2: Tracings of stopped-flow fluorescence assays of the hydrolysis of Dns-$(Gly)_3$-L-Phe, 2.5×10^{-4} M, catalyzed by zinc and cadmium carboxypeptidase A (Anson), 5×10^{-5} M, at pH 7.5 and 25° in 0.03 M Tris–1.0 M NaCl. Enzyme tryptophans were excited at 285 nm and their emission was measured by means of a band-pass filter peaking at 360 nm.

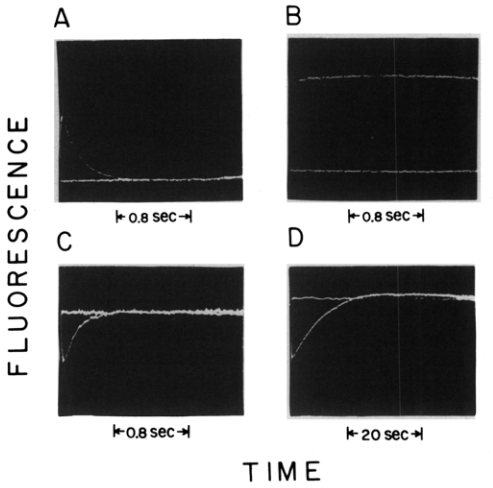

FIGURE 3: Stopped-flow fluorescence measurements of Dns-$(Gly)_3$-L-Phe, 1×10^{-4} M, binding to zinc (A,C) and apo-(B,D) carboxypeptidase A (Cox) at pH 7.5 and 25° in 0.03 M Tris–1.0 M NaCl. Concentration of the zinc and apoenzyme was 5×10^{-5} M. Enzyme tryptophans were excited at 285 nm. Dansyl emission (A,B) was measured by means of a 430-nm cut-off filter, and tryptophan emission (C,D) was measured by means of a band-pass filter peaking at 360 nm. Full scale deflection of fluorescence is 160 mV for all figures.

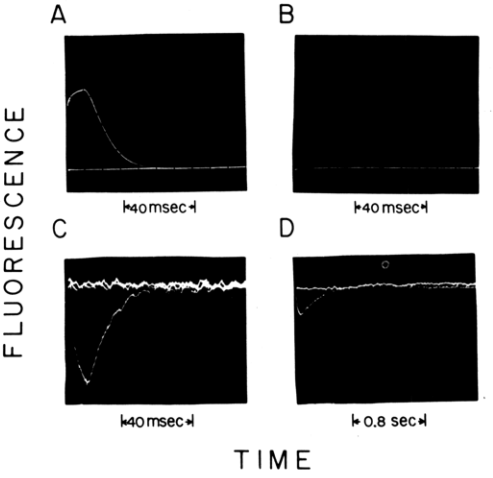

FIGURE 4: Stopped-flow fluorescence measurements of Dns-$(Gly)_3$-L-OPhe, 1×10^{-4} M, binding to zinc (A,C) and apo-(B,D) carboxypeptidase A (Cox). Concentration of the zinc and apoenzyme was 2.0×10^{-5} M. All other conditions are the same as in Figure 3. Full-scale deflections of fluorescence are 4.0 V (A,B) and 400 mV (C,D).

Mn > Cd, [(CPD)Co] being 150 times more active than [(CPD)Cd]. However, the corresponding association constants, K_m^{-1}, are essentially identical. On the other hand, metal substitution markedly alters the binding affinity of the exact ester analog, Bz-$(Gly)_2$-L-OPhe. The K_m^{-1} values decrease in the same order as do the k_{cat} values of peptide hydrolysis, *i.e.*, Co > Zn > Mn > Cd, the affinity of the ester for [(CPD)Co] being 30 times greater than for [(CPD)Cd]. Metal substitution, however, has no significant effect on the catalytic rate constant of ester hydrolysis.

Substrate Binding to the Apoenzyme. Stopped-flow fluorescence studies show that the apoenzyme binds the peptide, Dns-$(Gly)_3$-L-Phe, as tightly (Figures 3B and 3D) as does the Zn enzyme (Figures 3A and 3C); their initial E·S complex concentrations are nearly equal. However, the apoenzyme·peptide complex is stable and does not break down to products (Figure 3B) during the time needed for complete hydrolysis of the peptide by the zinc enzyme (Figure 3A). Hydrolysis of the peptide eventually does occur (Figure 3D), but at a rate which is proportional to the limiting amount of adventitious zinc present under the conditions of the assay. A tenfold increase in substrate concentration increases binding of the peptide to both the zinc and apoenzymes to the same extent. Hence, removal of the metal does not alter the affinity of the enzyme for the peptide.

The oscilloscope tracing shown in Figure 4A demonstrates the rapid enhancement in dansyl fluorescence following mixing of 1×10^{-4} M Dns$(Gly)_3$-L-OPhe and 2×10^{-5} M zinc enzyme. The increase in dansyl fluorescence during the "dead time" of the instrument proceeds to the identical maximal value which is observed during the time period apparent from the tracing in Figure 4A, demonstrating extremely rapid equilibration of enzyme and substrate to form the E·S complex. The decrease in the signal is a considerably slower process and reflects a diminution in enzyme-bound ester, as hydrolysis reduces the ester concentration. A complementary pattern is observed when quenching of enzyme tryptophan fluorescence by the dansyl group of the bound ester is measured (Figure 4C). The maximal changes in fluorescence are directly proportional to the equilibrium concentration of the E·S complex. Determination of these F_{max} values as a function of substrate concentrations allows the direct determination of the equilibrium dissociation constant, K_D (Auld *et al.*, 1972). For the ester, Dns$(Gly)_3$-L-OPhe, binding to the zinc enzyme a value of

TABLE II: Metallocarboxypeptidase-Catalyzed Hydrolysis of Bz-$(Gly)_2$-L-Phe and Bz-$(Gly)_2$-L-OPhe.[a]

	Bz-$(Gly)_2$-L-Phe		Bz-$(Gly)_2$-L-OPhe	
Metal	k_{cat} (min^{-1})	$10^{-3}K_m^{-1}$ (M^{-1})	$10^{-4}k_{cat}$ (min^{-1})	K^{-1} (M^{-1})
Cobalt	6000[b]	1.5[b]	3.9	3300
Zinc	1200	1.0	3.0	3000
Manganese	230[b]	2.8[b]	3.6	660
Cadmium	41	1.3	3.4	120

[a] Assays performed at 25°, pH 7.5, 1.0 M NaCl, and a buffer concentration of 0.05 M Tris for peptide hydrolysis and 10^{-4} M Tris for ester hydrolysis. [b] Values are for carboxypeptidase A (Cox) (Auld and Vallee, 1970a). All other values are for carboxypeptidase A (Anson).

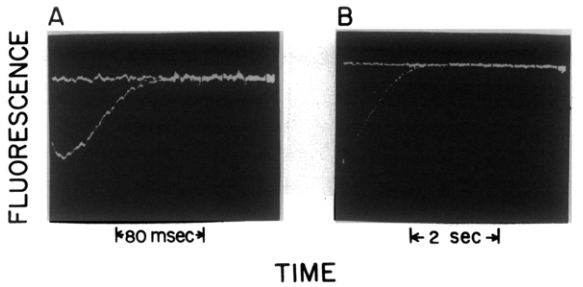

FIGURE 5: Binding of Dns-$(Gly)_3$-L-OPhe, 1×10^{-3} M, to zinc (A) and apo-(B) carboxypeptidase A (Cox) both at 5×10^{-5} M. Enzyme tryptophans were excited at 285 nm and their emission was measured by means of a band-pass filter peaking at 360 nm. Full-scale deflection of fluorescence is 160 mV for both figures. Measurements were made at pH 7.5 and 25° in 0.03 M Tris–1.0 M NaCl buffer.

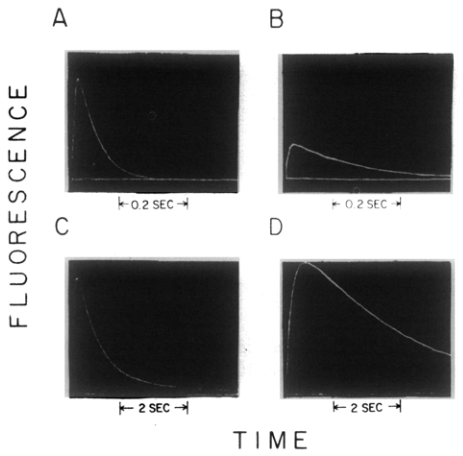

FIGURE 6: Phenyl acetate inhibition of carboxypeptidase A (Anson) catalyzed hydrolysis of Dns-$(Gly)_3$-L-OPhe, 4×10^{-5} M (A,B) and Dns-$(Gly)_3$-L-Phe, 1×10^{-4} M (C,D). Enzyme concentrations were 5×10^{-6} and 4×10^{-5} M for ester and peptide hydrolysis, respectively. Assays were performed in the absence (A,C) and presence (B,D) of 1×10^{-3} M phenyl acetate at 25° and pH 6.5, 0.03 M Mes–1.0 M NaCl. Excitation was at 285 nm and dansyl emission, above 430 nm, was observed. Full-scale deflections of fluorescence are 800 (A,B) and 160 mV (C,D).

2.5×10^{-5} M for K_D is obtained which is identical with that of K_m (Table I).

The hydrolysis of Dns-$(Gly)_3$-L-OPhe observed in the presence of apoenzyme again occurs at a rate close to that which can be attributed to the level of adventitious Zn^{2+} ions present. However, in contrast to the peptide, metal removal drastically reduces the binding of the ester, as indicated by the marked alteration in the initial fluorescent amplitudes (Figures 4B and 4D). The reduction of the dansyl fluorescence (Figure 4B) is even greater than that for the reduction of tryptophan quenching (Figure 4D). This additionally suggests that the dansyl quantum yield of the apoenzyme·ester complex is less than that of the zinc enzyme·ester complex. A tenfold higher ester concentration is required to observe identical quenching of tryptophan fluorescence in the zinc and apoenzyme·ester complexes (Figure 5). The extent of quenching of native enzyme tryptophan fluorescence by the ester increases only slightly, from 10 to 14%, as would be expected since the enzyme is close to full saturation over this substrate concentration range. In marked contrast over this same substrate concentration range the extent of quenching of apoenzyme tryptophan fluorescence by the ester increases markedly from 2 to 21%, indicating the apoenzyme binds the ester at least an order of magnitude more weakly than the zinc enzyme.[3]

Reversible Inhibition. The effect of an inhibitor on the fluorescent characteristics of an E·S complex readily identifies the mode of inhibition (Auld *et al.*, 1972). The behavior of phenyl acetate toward the native enzyme·Dns-$(Gly)_3$-L-OPhe complex at pH 6.5, for example, is characteristic of competitive inhibition (Figures 6A and 6B). In the presence of phenyl acetate, 1 mM, the initial peak height is reduced, but the area under the curve remains unchanged. The K_I was determined to be 3.2×10^{-4} M. However, when phenyl acetate acts on the E·S complex of the exact peptide analog Dns-$(Gly)_3$-L-Phe (Figures 6C and 6D), the area under the tracing increases greatly, consistent with a markedly reduced rate of hydrolysis. Since the inhibitor does not decrease the initial peak height, substrate binding has not been decreased. This is characteristic of noncompetitive inhibition. The K_I value, 3.3×10^{-4} M, is the same as that obtained for ester hydrolysis. Similar results have been obtained by steady-state means with a number of such inhibitors acting on peptide and ester pairs (Table III). Thus, while β-phenyl propionate and indole 3-acetate inhibit peptide hydrolysis noncompetitively, they inhibit ester hydrolysis competitively.

Discussion

Kinetic studies of carboxypeptidase A over the past two decades have revealed complexities in its catalytic behavior toward acylamino acids, dipeptides, and their ester analogs (Vallee *et al.*, 1968, 1970, and references cited therein). The varying degrees of activation and inhibition imposed by these substrates and their products of hydrolysis have precluded the determination of meaningful kinetic constants. A schematic model, based on multiple modes of substrate binding, has been proposed to accommodate the kinetic characteristics of carboxypeptidase toward these substrates (Vallee *et al.*, 1968). It incorporates nonidentical but overlapping binding of dipeptides and analogous esters as the basis for the kinetic anomalies. The model suggested that increasing the length of these substrates would eliminate anomalous binding and likely normalize their enzymatic hydrolysis. Kinetic studies of both oligopeptides and their ester analogs has, indeed, borne out this prediction (Auld and Vallee, 1970a; Auld and Holmquist, 1972, Figure 1). However, the question has remained whether or not the native and modified enzymes which discriminate between peptide and ester substrates displaying anomalous kinetics (*e.g.*, Cbz-Gly-L-Phe and Bz-Gly-L-OPhe) would also differentiate between members of peptide–ester pairs obeying Michaelis–Menten kinetics. This present study of oligopeptides and their depsipeptide analogs now answers this question. We present here several lines of evidence which indicate unequivocally that the overall mechanisms of the carboxypeptidase A catalyzed ester and peptide hydrolysis must differ.

Studies using peptides devoid of anomalous kinetics have clearly shown that carboxylic acids such as β-phenyl propio-

[3] The fractional quenching of enzyme tryptophan fluorescence in the E·S complex, ϕ, is equal to $T[ES]/[E_T]$ where $[ES]/E_T]$ is the fraction of enzyme complexed with substrate and T is the energy transfer from the dansyl group of the substrate in the E·S complex to the tryptophans of the enzyme. For a given enzyme and substrate, T will be a constant. A change in ϕ as a function of substrate concentration, therefore, means the ratio of $[ES]/[E_T]$ is changing.

nate and phenyl acetate inhibit peptide hydrolysis noncompetitively (Auld and Vallee, 1970a; Auld *et al.*, 1972). The present studies demonstrate that, remarkably, these same agents are competitive inhibitors of the exact ester analogs of these peptides (Figure 6 and Table III). The identical K_I values obtained for both classes of substrates suggest the formation of identical enzyme·inhibitor complexes in the presence both of ester and peptide substrates. However, the differences in modes of inhibition are incompatible with the postulate that esters and peptides bind in an identical manner. Other recent inhibition studies also indicate that esters and peptides must bind differently to the native enzyme. Thus, the D enantiomer of Bz-Gly-OPhe does not inhibit hydrolysis of tripeptides but competitively inhibits hydrolysis of their depsipeptide analogs (Lange *et al.*, 1974).

Substitution of Cd for Zn first demonstrated a difference in the esterase and peptidase activities of the enzyme (Coleman and Vallee, 1960). The activity of [(CPD)Cd] toward Bz-Gly-L-OPhe is increased, but the enzyme is virtually inactive toward Cbz-Gly-L-Phe. However, Cbz-Gly-L-Phe inhibits this esterase activity indicating that the cadmium enzyme can still bind the peptide (Vallee *et al.*, 1968).

Stopped-flow fluorescence studies of E·S complexes have now provided a direct comparison of the peptide binding affinities of the zinc and cadmium enzymes and, simultaneously, an explanation for the different roles of metals in peptide and ester hydrolysis. [(CPD)Cd] binds the peptide Dns-$(Gly)_3$-L-Phe as readily as does [(CPD)Zn] but catalyzes its hydrolysis at a rate which is considerably reduced (Figure 2). Steady-state rate studies of oligopeptides are in agreement with this observation (Table I). The catalytic rate constants of the Cd enzyme are markedly decreased for all peptides examined, but the association constants (K_m^{-1} values) of the Cd enzyme are identical with those of the Zn enzyme (Table I). However, in marked contrast for all esters examined the catalytic rate constants of the Cd enzyme are nearly the same as those of the Zn enzyme while the association constants of the Cd enzyme are greatly decreased (Table I).

It has been shown previously that dipeptide substrates of native carboxypeptidase interact with the apoenzyme and thereby prevent restoration of activity by metal ions (Felber *et al.*, 1962) but that the ester, Bz-Gly-OPhe, does not (Coleman and Vallee, 1962a). These kinetic experiments served as the basis for a physicochemical approach employing gel filtration to detect the existence of apoenzyme·peptide complexes and to measure their binding constants (Coleman and Vallee, 1962a,b). By this means free and amino blocked dipeptide substrates were shown to prevent the association of the metal ion with the apoenzyme (Coleman and Vallee, 1962a).

Gel filtration studies allow determination of binding constants of apoenzyme·peptide complexes, but not those of the zinc enzyme·peptide complexes due to the rapid hydrolysis of the peptide by the zinc enzyme. In addition, the anomalous kinetics of the dipeptides makes it difficult to compare peptide dissociation constants for the apoenzyme with their respective K_m values for the zinc enzyme. However, stopped flow fluorescence studies of E·S complexes of substrates displaying Michaelis–Menten kinetics have now overcome both of these problems. Thus, the apoenzyme, although unable to catalyze the hydrolysis of peptide substrates, binds them to the same degree as does the zinc enzyme (Figure 3). Binding of the exact ester analog, however, decreases by an order of magnitude when zinc is removed from the native enzyme (Figures 4 and 5). Hence, the binding of peptides to metallocarboxypeptidases must differ from that of esters.

TABLE III: Carboxylic Acid Inhibitors of Ester and Peptide Hydrolysis.[a]

Substrate	Inhibitor	10^4K_I (M)	Type of Inhibition
Bz-$(Gly)_2$-L-Phe[b]	β-Phenyl propionate	1.2	Noncompetitive
Bz-$(Gly)_2$-L-OPhe	β-Phenyl propionate	1.2	Competitive
Cbz-$(Gly)_2$-L-Phe[b]	Indole 3-acetate	1.7	Noncompetitive
Bz-$(Gly)_2$-L-OLeu	Indole 3-acetate	1.6	Competitive
Dns-$(Gly)_3$-L-Phe	Phenyl acetate	3.3	Noncompetitive
Dns-$(Gly)_3$-L-OPhe	Phenyl acetate	3.2	Competitive

[a] Assays performed at 25°, pH 7.5, 1.0 M NaCl, 0.05 M Tris except for the phenyl acetate study where the conditions were pH 6.5, 1.0 M NaCl, 0.03 M Mes. [b] Results of Auld and Vallee (1970a).

A positively charged residue in the active center has long been thought to be a major determinant of the specificity of carboxypeptidase for which a free C-terminal carboxyl group of the substrate is mandatory (Waldschmidt-Leitz, 1931; Smith, 1949; Vallee *et al.*, 1963). Since acylation experiments have excluded lysines (Riordan and Vallee, 1963), it seemed possible that an arginyl residue might function as the binding locus for the carboxylate group (Vallee and Riordan, 1968). From X-ray crystallographic studies of the Gly-L-Tyr complex of the crystalline enzyme it has since been concluded that the carboxyl group of this pseudosubstrate is bound to Arg-145. It has been inferred further that this arginine is the enzyme specificity determinant for interaction with the C-terminal free carboxyl group of *all* substrates and is thus indispensable to catalysis by carboxypeptidase (Lipscomb *et al.*, 1968). Chemical modification of arginine in carboxypeptidase diminishes the rate of Cbz-Gly-L-Phe hydrolysis, consistent with the hypothesis that peptide substrates can bind to an arginyl residue (Riordan, 1973). However, this modification does not diminish esterase activity toward Bz-Gly-L-OPhe suggesting that a residue other than arginine serves as the recognition site and binding locus for the free carboxyl group of esters.

Direct binding of substrates to the metal through their carboxyl group was first suggested by Lumry and Smith (1955). In view of the present results, the active-site metal may indeed serve as a primary binding locus for esters, but such binding appears unlikely for peptides. Thus, metal substitution for zinc at the active site profoundly affects the binding of esters to the enzyme (Co > Zn > Mn > Cd) but not the rate at which they are hydrolyzed. In contrast, the same series of metals profoundly affects the rate determining step in the hydrolysis of peptides (Co > Zn > Mn > Cd) but not their association constants (Tables I and II). Moreover, the apoenzyme binds peptides as tightly as the zinc enzyme but binds esters much more weakly. These results would be expected if the metal atom were to interact with the carboxyl group of esters during the binding step

but with the carbonyl group of peptides during the catalytic step.

Inhibition studies with carboxylate inhibitors such as phenyl acetate and β-phenyl propionate which are thought to bind to the metal further support this hypothesis. These inhibitors compete with the binding of esters but not peptides (Figure 6 and Table III). Direct binding studies and metal ion exchange data led Coleman and Vallee (1962b, 1964) to propose that binding of β-phenyl propionate involves a metal–carboxyl group interaction. Crystallographic studies with *p*-iodo-β-phenyl propionate (Lipscomb *et al.*, 1968) and nuclear magnetic resonance (nmr) studies of manganese carboxypeptidase interacting with β-phenyl propionate (Navon *et al.*, 1968, 1970) have since confirmed this postulate. Presumably, the carboxyl groups both of esters and of such carboxylic acid inhibitors compete for the metal. Peptides seem to bind to the guanido group of an arginyl residue (Riordan, 1973).

It is remarkable that the enzyme binds esters and peptides differently even though the two types of substrates vary only in the susceptible bond. X-Ray diffraction studies of the binding of ester·substrates or ester·pseudosubstrates to the crystalline enzyme have not been successful. However, a coordinated series of conformational changes is thought to occur when the peptide pseudosubstrate, Gly-Tyr, binds to the crystalline enzyme (Lipscomb *et al.*, 1968). The X-ray structure analysis of the *nonproductive* enzyme·Gly-Tyr complex led to the suggestion that an initial interaction of the carboxyl group of Gly-Tyr with Arg-145 brings about a substrate-induced conformational change of 12 Å in Tyr-248 so that the phenolic hydroxyl group would move into the vicinity of the susceptible bond of the substrate (Lipscomb *et al.*, 1968). Two facts are at variance with this interpretation. First, recent findings have shown that Tyr-248 can be coordinated to the active-site metal *in the absence of substrate*. Thus, the properties of the enzyme modified with diazotized arsanilic acid have demonstrated that Tyr-248 can be coordinated to the metal in the absence of substrate (Johansen and Vallee, 1971, 1973; Johansen *et al.*, 1972). These findings have led to a reexamination of earlier X-ray data (Lipscomb *et al.*, 1968) and their reinterpretation now uncovers and confirms the existence of such a zinc·Tyr-248 interaction in the native X-ray crystals (Lipscomb, 1973). Second, the proposed substrate-induced conformational changes of Tyr-248, initiated by interaction of the Gly-Tyr carboxyl group with Arg-145, are inconsistent with the difference in ester and peptide binding. If this were the mechanism by which *productive* peptide complexes are formed, it would be difficult to understand why hydrolysis of their structurally exact ester analogs (*e.g.*, Dns-$(Gly)_3$-L-Phe and Dns-$(Gly)_3$-L-OPhe) should not follow both the same binding and catalytic pathways.

Since these two types of substrates do bind differently, however, it is likely that the first step in their respective binding processes involves the ester *vs.* the amide bond, the only chemical feature which distinguishes these esters and peptides. It would seem that either the stereochemistry about the ester bond differs sufficiently from that about the amide bond so as to prevent it from binding in the same manner as peptides or the NH of the susceptible amide bond may be critical to the initiation of peptide binding. Thus, the NH group of the peptide, *e.g.*, might interact either with Tyr-248 or with the metal serving as the initial loci for peptide binding. In this regard, studies of azocarboxypeptidase in solution have shown that binding of Gly-L-Tyr breaks up the azo-Tyr-248·Zn complex (Johansen and Vallee, 1971). It is quite conceivable then that binding of the peptide leads directly to an interaction of the NH of the susceptible bond with the phenolic group of Tyr-248, which simultaneously orients the peptide carboxyl group toward Arg-145 and its carbonyl toward the zinc atom. Such circumstances would then lead one to expect differences between the binding of esters and peptide substrates. Lacking the potential of this interaction would leave the esters free to bind in a different manner and might then even allow for the participation of different catalytic groups in the hydrolysis of the two types of substrates. The present data clearly call for a reexamination of current views regarding the mechanism of action of carboxypeptidase A.

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Functional Arginyl Residues as NADH Binding Sites of Alcohol Dehydrogenases†

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ABSTRACT: Two specific arginyl reagents, 2,3-butanedione and phenylglyoxal, inactivate the alcohol dehydrogenases from human liver, horse liver, and yeast. These arginyl residues have been identified as components of the NADH binding sites in these three enzymes as substantiated by the protective effect of NADH against loss of activity. Coenzyme binding to the modified horse enzyme is virtually abolished as revealed by absorption and circular dichroic spectroscopy and gel filtration studies. Protection experiments with a series of coenzyme analogs further indicate that these arginyl residues interact, most likely, with the pyrophosphate moiety of the coenzyme. Specific, reversible modifications of the arginyl residues by butanedione and of the catalytically essential thiol residue, Cys-46, by *p*-mercuribenzoate differentiate arginine from cysteine modification. Moreover, differences in the rates of specific irreversible carboxymethylation of Cys-46 by iodoacetate in native and butanedione-modified liver alcohol dehydrogenase indicate that the functional arginyl residues are in close proximity to the active center cysteines.

At least 1000 of the more than 1500 enzymes now known act on negatively charged substrates or require anionic cofactors. As part of a general study on the mode of binding of such ligands to these enzymes, we have found that arginyl residues often serve as the complementary, positively charged recognition sites. Thus, a single arginyl residue participates in binding the terminal carboxyl group of peptide substrates to carboxypeptidase A (Vallee and Riordan, 1968; Riordan, 1970, 1973) and arginines are critical for substrate binding to *Escherichia coli* alkaline phosphatase (Daemen and Riordan, 1974). Moreover, metabolic regulation of enzyme function by hormones or other means frequently involves interaction with phosphate, cyclic AMP, ATP, their derivatives, or related compounds. Recognition of these anionic ligands might constitute a very important function of arginyl residues in proteins.

The present study examines such concepts particularly as they may pertain to binding of NADH to the alcohol dehydrogenases. We have studied the role of arginine in binding this coenzyme to the enzymes from human liver, horse liver, and yeast. Arginine-specific, α-dicarbonyl reagents inactivate all three of these dehydrogenases, and loss of activity correlates both with arginine modification and loss of coenzyme binding. These results identify arginyl residues as NADH binding sites in these alcohol dehydrogenases and may bear on the binding of NADH and other nucleotide coenzymes to enzymes, in general.

Materials and Methods

Horse liver alcohol dehydrogenase (Boehringer-Mannheim Corporation) was obtained as a crystalline suspension in 0.02 M phosphate buffer (pH 7.5) and 10% ethanol. Concentrated enzyme solutions were prepared by dialyzing the suspension for 3 days *vs.* daily changes of a 1000-fold volume excess of 0.1 M phosphate (pH 7.5, 4°); centrifugation at 5000 rpm for 15 min, 4°, removed insoluble material. Protein concentration, using a molar absorptivity at 280 nm of 3.57×10^4 M^{-1} cm^{-1}, and enzymatic

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