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Phil. Trans. R. Soc. Lond. B 1970 **257**, 215-230
doi: 10.1098/rstb.1970.0021

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Phil. Trans. Roy. Soc. Lond. B **257**, 215–230 (1970) [215]
Printed in Great Britain

Chemical approaches to the mode of action of carboxypeptidase A

By B. L. Vallee, J. F. Riordan, D. S. Auld and S. A. Latt
Biophysics Research Laboratory, Department of Biological Chemistry, Harvard Medical School, U.S.A. and the Division of Medical Biology, Peter Bent Brigham Hospital, Boston, Massachusetts, U.S.A.

Our studies of the mechanism of action of carboxypeptidase A have been guided by the dual specificity of this enzyme as altered either by replacement of the native zinc atom at the active site with other metal ions or by chemical modification of amino acid side chains. In almost all instances esterase activity toward HPLA† increased and peptidase activity toward CGP decreased in response to such treatments. A mechanism postulating the modification of a single catalytically active amino acid residue was designed as the simplest chemical event that could provide a unified account for such similar functional consequences in response to a variety of agents (Vallee, Riordan & Coleman 1963). The mechanism was postulated before the identification of the residues affected by chemical modifications. Similarly, the number and nature of binding groups were unknown, and thus, could not be considered. It was recognized that the scheme would have to be amplified, once specific residues were identified. Significant progress, in this regard, has occurred over the past several years and this report summarizes the present status of our understanding.

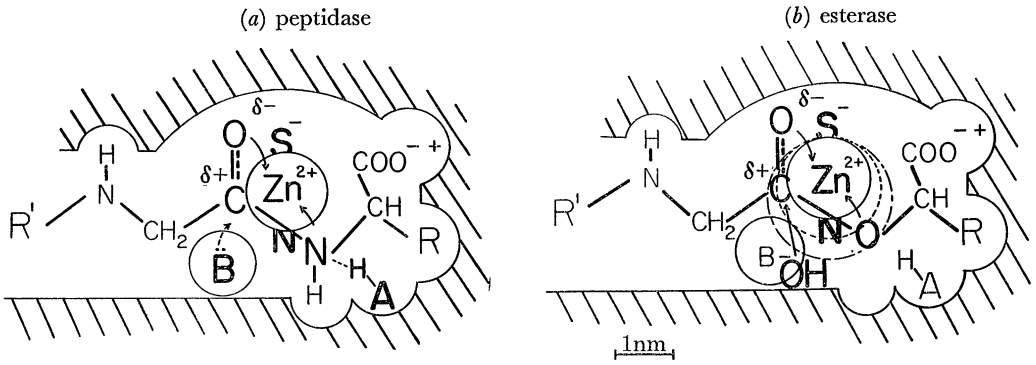

Figure 1. (*a*) Mechanism of peptide hydrolysis. Alternative possibilities could include a water molecule between group B and the carbonyl carbon. (*b*) Mechanism of ester hydrolysis: the relative ionic radii of Zn^{2+}, Cd^{2+} and Hg^{2+} are indicated.

The original mechanistic scheme (Vallee *et al.* 1963) is presented in figure 1. Binding and specificity studies with peptides, esters and inhibitors (Coleman & Vallee 1962*a*, *b*) had demonstrated the importance of the R group and the two —NH— functions of the substrate. The effect of peptides, peptide amides and inhibitors on the restoration of zinc to apocarboxypeptidase indicated that the susceptible peptide bond be placed near the zinc atom, with the C-terminal carboxyl group interacting with a positive charge.

Peptide hydrolysis was thought to be initiated by the nucleophile, group B, facilitated by the

† Abbreviations: HPLA, hippuryl-DL-phenyllactate; CGP, carbobenzoxyglycyl-L-phenylalanine; DFP, diisopropylphosphofluoridate; TNM, tetranitromethane; DHT, 5-diazo-1*H*-tetrazole; BGGP, benzoylglycylglycyl-L-phenylalanine; CGGP, carbobenzoxyglycylglycyl-L-phenylalanine; CGGGP, carbobenzoxyglycylglycylglycyl-L-phenylalanine; BG, benzoylglycine; CG, carbobenzoxyglycine; CGGG, carbobenzoxyglycylglycylglycine.

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electron withdrawing effect of the metal atom on the oxygen of the carbonyl. A second, acidic group in the active centre, AH, was postulated to donate a proton to the peptide nitrogen of the C-terminal residue. The 'one catalytic residue mechanism' could account for the curtailment of dual specificity by various acylating agents and metal substitutions by assuming that group B, essential for the hydrolysis of the peptide actually retarded the hydrolysis of the ester. The decrease in peptidase and increase in esterase activities could then be attributed to the elimination of group B by the various modifications. However, it was clear that any realistic proposal had to be founded on more complete information as to the nature and actual number of catalytic and binding groups which would have been entirely a matter of speculation at that juncture (Vallee *et al.* 1963).

Soon after the introduction of this scheme we found the effects of acylation attributable to the modification of two tyrosyl residues (Simpson, Riordan & Vallee 1963; Riordan & Vallee 1963). It could not be ascertained whether only one or both of these functioned in either activity and/or substrate binding. It was pointed out explicitly that neither one of the tyrosyl residues could be equated with group A or B in the above mechanism (Vallee 1964*a*).

The proposed mechanism continued to serve as a working hypothesis for further experimentation. Efforts were directed to discern, by chemical means, the groups involved in either catalysis or binding, and from such investigations together with kinetic studies a dual site model for peptide and ester binding evolved (Vallee 1967; Vallee, Riordan, Bethune, Coombs, Auld & Sokolovsky 1968). In addition, experiments directed toward understanding the role of the metal in the catalytic process were continued.

TABLE 1. ACTIVITIES OF METALLOCARBOXYPEPTIDASES

	peptidase† (c)	esterase‡ ($k \times 10^{-3}$)
apo	0	0
zinc	34	7
cobalt	68	8
nickel	16	3
manganese	9	11
cadmium	0	10
mercury	0	6
rhodium§	0	5
lead	0	4
copper	0	0

† 0.02 mol l^{-1} of CGP, 273 K, pH 7.5.
‡ 0.01 mol l^{-1} of HPLA, 298 K, pH 7.5. Methods of assay are discussed in Davies *et al.* (1968).
§ G. Rouschias (unpublished observations).

The enzymic consequences of replacing cobalt, manganese, nickel, cadmium, mercury, lead, rhodium, and copper for the native zinc atom of carboxypeptidase (Coleman & Vallee 1960) are summarized in table 1. Exchange experiments suggested mutually exclusive metal binding sites (Coleman & Vallee 1961). The peptidase activity of the cobalt enzyme is greater than that of the zinc enzyme over a large substrate concentration range (Davies, Riordan, Auld & Vallee 1968). In contrast, the cadmium, mercury, rhodium and lead enzymes, while esterases, are not peptidases. Copper and a large number of other metals tested are completely inactive toward both CGP and HPLA. Significantly, perhaps, the esterase activity of the cadmium enzyme was inhibited by peptide substrates, suggesting that these still bind to the cadmium enzyme though they are not hydrolysed (Vallee *et al.* 1968). In accord with the intent to reduce all activity

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changes to a single, catalytically important residue as a simple scheme, the curtailment of the dual specificity of the cadmium and mercury enzymes was related to their atomic radii known to differ significantly from that of zinc (Vallee *et al.* 1963). The spectral probe characteristics of these metals are unfavourable, of course, and the known differences in the geometry of their complexes could not be determined in the enzyme.

Cobalt, however, is known to be a good spectral probe for the investigation of its ligand environment, as was apparent from early absorption spectra of the cobalt enzyme (Coleman & Vallee 1960). Improvements in experimental facilities have now permitted more detailed investigations of its absorption and ellipticity spectra. The spectra of cobalt carboxypeptidase are quite unusual when compared with those of simple cobalt ions, as has been observed in the case of spectra of other enzymes in which cobalt could be substituted for zinc with retention of enzymic activities (Vallee & Williams 1968*a*). These two ions are known to replace one another readily in zinc enzymes, perhaps because of their capacity to accept similar ligand geometries (Vallee & Williams 1968*b*).

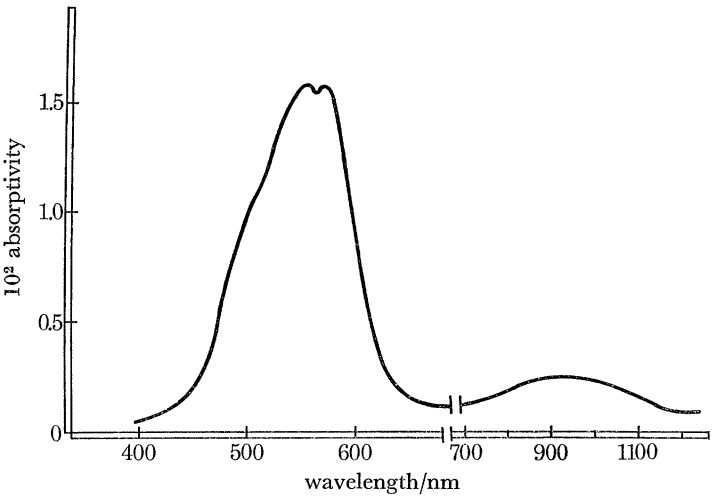

FIGURE 2. Absorption spectrum of cobalt carboxypeptidase A_α (1 mmol l^{-1}), in 1 mol l^{-1} of NaCl, 5 mmol l^{-1} of Trischloride, pH 7.1, at 293 K. Each mole of enzyme contained 0.97 ± 0.05 mol atomic cobalt.

The cobalt carboxypeptidase absorption spectrum has maxima [absorptivities] at 555 [*ca.* 150] and 572 [*ca.* 150], and 940 nm [*ca.* 25], and a shoulder at about 500 nm [110]. Neither in location of bands nor in intensities is this spectrum identical with those of octahedral or tetrahedral cobalt complex ions (figure 2). The maxima found at 555 and 572 nm at 293 K split at 4.2 K. While the absorptivities of these two maxima remain the same relative to one another at 4.2 K the maximum at 555 shifts to 532 nm, and shoulders at 505 and 480 become distinctive. Glycyl-L-tyrosine addition at 293 K results in maxima at 510 and 550 nm. On addition of glycyl-L-tyrosine the major, negative Cotton effect of the cobalt enzyme at 538 nm is inverted, becoming positive with a peak at 553 nm. The effect of β-phenylpropionate is shown for comparison in figure 3 (Latt & Vallee 1969). A number of other inhibitors have different spectral effects. The ellipticity spectra demonstrate asymmetry of the cobalt environment, indicative of an irregular geometry, as already implied by the absorption spectra. A change in circular dichroism following glycyl-L-tyrosine addition has not been considered previously as a mode of perturbation of the active site of carboxypeptidase and of its environment. Such changes may or may not reflect a change in protein conformation.

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Dissimilarities between copper and iron complex ions on one hand, and copper and non-heme iron enzymes on the other, have been pointed out also. The lack of correspondence between the spectra of such metalloenzymes, in the absence of substrate, with presently known models extends to other physical-chemical criteria. The recognition of these phenomena has led to the suggestion that the active sites of metalloenzymes are in an entatic state, i.e. in a state of tension

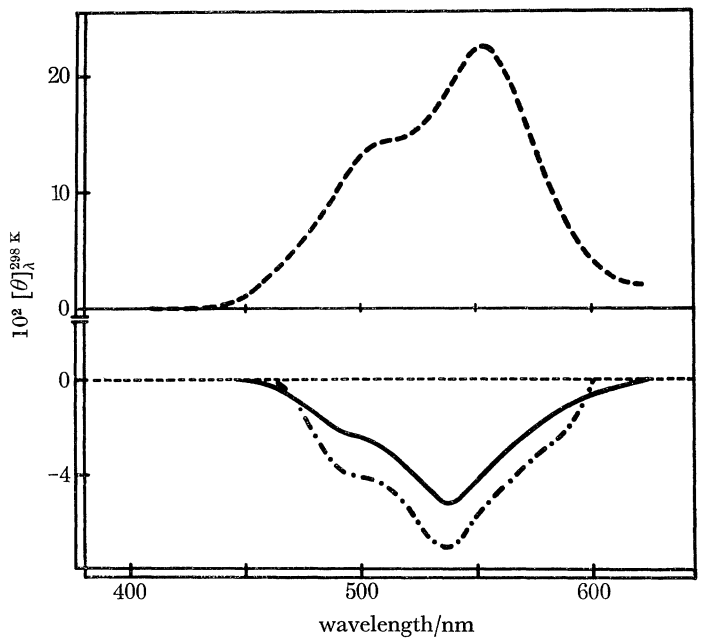

FIGURE 3. Effect of 10 mmol l^{-1} of glycyl-L-tyrosine (---) and 2 mmol l^{-1} of β-phenylpropionate (- - - -) on the circular dichroism of cobalt carboxypeptidase A_α (——) at 298 K. Preparation and properties of the enzyme as in figure 2.

or stretch and perhaps possessing an energy close to that of a transition state, constituting an energetically poised domain (Vallee & Williams 1968*a*, *b*). Such considerations could pertain to carboxypeptidase. On this basis, the difference between the physical properties of metals in such metalloenzymes and in models could be understood. These physical properties of metalloenzymes might reflect geometries characteristic of unstable intermediates of chemical reactions. Simple metal complex ions, which have generally served as models for metalloenzymes, would have more stable geometries. To ensure meaningful comparisons, the future design of suitable models for metalloenzymes should incorporate the very characteristics of metal complexes occurring in metalloenzymes.

These considerations may be pertinent to some aspects of the chemistry of the metal binding site of the enzyme which have been discussed fully very recently (Vallee & Riordan 1968). Studies of the metal binding site of carboxypeptidase were based on the assumption that its metal ligands and those of metalloenzymes might have reactivities comparable to those of amino acid and peptide models. The physical chemical properties suggesting the entatic nature of active sites of metalloenzymes in general, and of cobalt carboxypeptidase in particular, mitigate this assumption. Only the properties of bidentate models were available for comparisons with metalloenzymes (Coleman & Vallee 1961; Vallee, Williams & Coleman 1961); the properties of similarly suitable series of tri- and multidentate complex ions were unknown and, in fact, have not been studied in sufficient detail as yet. However, reversible metal binding to

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apoprocarboxypeptidase appeared to be a system, similar to if not identical with apocarboxypeptidase, which seemed capable of extending such comparisons. The zymogen–enzyme pair was thought to provide a useful parallel in an attempt to join pertinent factors of protein and coordination chemistry. Indeed, binding of a series of metal ions proved weaker in the zymogen than in the enzyme, and suggested one more ligand to the metal in the enzyme than in the zymogen (Piras & Vallee 1967). Chemical modifications of the enzyme were performed in the presence and absence of zinc to discern the nature of its ligands (Coombs, Omote & Vallee 1964). The results of such studies (table 2) together with comparisons of the sequence and

Table 2. Reactions of native and zinc free carboxypeptidase

	Ag^+	pMB	$Fe(CN)_6^{3-}$
	(moles reagent/mole carboxypeptidase)		
native enzyme	0	0	0
zinc free enzyme	0.91	0.95	1.22

magnitudes of stability constants of bidentate metal complex ions with those of a series of metallocarboxypeptidases—assuming the site to be bidentate—suggested nitrogen–sulphur ligands of the enzyme (Coleman & Vallee 1961; Vallee *et al.* 1961). Thus, apocarboxypeptidase A reacted with Ag^+, *p*-mercuribenzoate and ferricyanide but not with iodoacetate, iodoacetamide or *N*-ethylmaleimide. The zinc enzyme reacted with none of these (Vallee, Coombs & Hoch 1960; Coombs *et al.* 1964). Analogous results with procarboxypeptidase were obtained (Piras & Vallee 1967). Carboxypeptidase B reacted with Ag^+, *p*-mercuribenzoate, but, in addition, with iodoacetamide, *N*-ethylmaleimide and *N*-4-(dimethylamino-3,5-dinitrophenyl)maleimide (Wintersberger, Neurath, Coombs & Vallee 1965).

Table 3. Complementarity of Ag^+ titratable groups and zinc content of procarboxypeptidase A and carboxypeptidases A and B†

	Zn^{2+}	Ag^+ titre	sum
(PRO-CPD)-A	0.09	0.78	0.87
	0.56	0.38	0.94
	0.92	0.06	0.98
(CPD)-A	0.07	0.84	0.91
	0.57	0.42	0.99
	1.0	0	1.0
(CPD)-B	0.08	0.87	0.95
	0.38	0.76	1.14
	1.08	0	1.08

† For experimental details see Piras & Vallee (1967), Vallee *et al.* (1960), Coombs *et al.* (1964) and Wintersberger *et al.* (1965).

Table 3 demonstrates the results of interacting apoprocarboxypeptidase A, apocarboxypeptidase A and apocarboxypeptidase B with Ag^+ in the presence and absence of zinc. The molar sums of Zn^{2+} and of a group titrating with Ag^+ is unity in these three proteins, suggesting the presence of the same group, thought to be a thiol group, in all instances, based on the known specificities of the methods employed.

The X-ray data do not reveal a thiol group at the active site of carboxypeptidase A_α, and a tridentate site consisting of His, Glu, and Lys (or Glx) has been reported (Lipscomb *et al.*

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1968), calling for a re-evaluation of the data obtained with chemical methods. The presumed specificities of these methods do not suggest obvious conclusions as to the nature of the reacting residue(s) (Boyer 1959).† It is interesting that the three different but functionally related proteins exhibit similar chemical reactivities at their metal binding sites while utilizing combinations of ligands which can mimic to varying degrees the reactivity of sulphur in model systems. The basis for these observations is quite unknown. However, it could be that owing to environmental effects the metal ligands exhibit unusual chemical reactivities, analogous perhaps to those observed for amino acid side chains involved in catalysis (Oosterbaan & Jansz 1965). The metalloenzyme spectra, thought to reflect irregular coordination geometries, might be an expression of this circumstance as well. In fact, to date, chemical means have not identified the complete metal binding sites of any metalloenzyme (Vallee & Wacker 1969), and the present ambiguities may but signal new insight regarding the manner in which metalloenzymes perform their function.

The structural features of metalloenzymes, manifesting in unusual physical properties of their complexes may also determine the chemical reactivities of the participating ligands themselves. Such a hypothesis might serve as a basis for integration of the data obtained by chemical and X-ray studies in this particular case. There is good precedent for unusual reactivity toward specific reagents of active centre amino acid residues. Thus, in serine enzymes only the active centre seryl residue is reactive toward, e.g. DFP. In these instances the explanation is still being sought (Hartley, this volume, p. 77).

Such reactivity has also been observed on chemical modifications of amino acid side chains of carboxypeptidase. The alteration of peptidase and esterase activities by a number of reagents (Vallee *et al.* 1963) incurred a search for the groups modified in order to elucidate features of the mechanism and of substrate binding. The realization that tyrosyl residues participate in the catalytic function of carboxypeptidase was the consequence (Vallee 1964*a*). Acetylation with mono- and dicarboxylic acid anhydrides and *N*-acetylimidazole (Simpson *et al.* 1963; Riordan

TABLE 4. CHANGES IN PEPTIDASE AND ESTERASE ACTIVITIES* ON MODIFICATION OF FUNCTIONAL RESIDUES IN CARBOXYPEPTIDASE A

reagent	percentage control activity		functional residue modified
	peptidase	esterase	
acetyl imidazole	< 2	700	Tyr
acetic anhydride	< 2	610	Tyr
iodine	< 2	500	Tyr
tetranitromethane	14	190	Tyr
5-diazo-1*H*-tetrazole (8 ×)	90	180	Tyr
5-diazo-1*H*-tetrazole (45 ×)	5	180	His
2,3-butanedione	11	240	Arg
cyclohexyl-3-(2-morpholino-ethyl)carbodiimide	12	9	carboxyl

* Conditions as in table 1.

& Vallee 1963), nitration with tetranitromethane, TNM (Sokolovsky, Riordan & Vallee 1966), iodination (Simpson & Vallee 1966) and coupling with 5-diazo-1*H*-tetrazole (DHT) (Sokolovsky & Vallee 1967) or *p*-azobenzenearsonate (Kagan 1968) have all been shown to alter esterase and peptidase activities due to tyrosyl modification (table 4). Most of these have qualitatively

† Many preparations of carboxypeptidase γ and δ contain more than 5 mol of atomic sulphur, i.e. between 6 and 7 mol of atomic sulphur, not accounted for by amino acid analysis. The chemical identity and source of this sulphur is presently unknown (Vallee & Riordan 1968).

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similar effects on function: esterase activity increases and peptidase activity decreases concomitantly. Two of the 19 tyrosyl residues of the enzyme appear to be unusually reactive (Simpson *et al.* 1963; Riordan & Vallee 1963). Depending on the particular reagent employed, one, the other or both of these residues are modified. Inhibitors, such as β-phenylpropionate and substrates protect against modification and the consequent activity changes.

A number of experiments employing successive chemical modifications with different 'tyrosyl' reagents have examined a possible role of each of the two tyrosyl residues in function (Riordan, Sokolovsky & Vallee 1967*a*). Prior acetylation completely prevents azocoupling or nitration. Hence, the tyrosyl residues modified by DHT and TNM would seem to be among those which are acetylated. Conversely, nitration of one tyrosyl residue prevents the functional consequences of acetylation. Azocoupling with DHT followed by nitration does not affect the esterase activity of azocarboxypeptidase. Spectral analysis of the product reveals the presence of one azotyrosyl and of one nitrotyrosyl residue each per mole. This suggests that under these circumstances enzymic changes due to azocoupling on one hand and to nitration on the other may result from modification of different tyrosyl residues. Hence, these tyrosyl residues could play a different role in the mechanism of action of the enzyme (Riordan *et al.* 1967*a*). Work is in progress in collaboration with Dr Neurath's laboratory to identify the location of these residues in the primary sequence of the enzyme. A peptide containing one of the active centre tyrosyl residues has been isolated from iodocarboxypeptidase (Roholt & Pressman 1967) and has been located in the X-ray structure of carboxypeptidase by Lipscomb *et al.* (1968).

Coupling of carboxypeptidase with a 45-fold molecular excess of DHT abolishes the peptidase activity of monoazotyrosylcarboxypeptidase, while its esterase activity is virtually unaltered (Sokolovsky & Vallee 1967). The decrease of peptidase activity correlates with the coupling of one histidyl residue. Although the data do not indicate in what manner coupling of a histidyl residue might bring about the loss of peptidase activity, they suggest that the integrity of such a residue may be essential to some phase of the catalytic process (Sokolovsky & Vallee 1967).

These results of chemical modification should be viewed in the light of kinetic evidence implicating histidine in the mechanism of action of carboxypeptidase. The inflexion point at about pH 6.7 of the pH rate profile for the hydrolysis of CGP was attributed to the ionization of a histidyl residue (Neurath & Schwert 1950). Recent studies on the temperature and pH dependence of V_{max} for the hydrolysis of the peptide substrates BGGP and CGGP demonstrate a group ionizing with a pK' of 6.2 and yield heats of ionization between 25 and 30 kJ mol^{-1} (Auld 1969). Based on models, such values are consistent with, though not necessarily diagnostic of, a functional histidyl residue (Bruice & Schmir 1959). The possibility of an unusual carboxyl group must also be considered.

Our initial mechanism postulated that the C-terminal carboxyl group specificity requirement of carboxypeptidase might be due to an interaction of this group with a positive charge, e.g. a lysyl or arginyl residue on the protein surface (figure 1). While there were adequate chemical methods for the modification of amino groups, attempts to implicate the direct participation of lysyl residues in activity have been unsuccessful. Acylation with mono- and dicarboxylic acid anhydrides or amidination with methylacetimidate modified 11 of the 15 lysyl residues, but these modifications were virtually without effect on either esterase or peptidase activities (Riordan & Vallee 1963, 1964; Sokolovsky & Vallee 1967).

Suitable methods for the modification of arginyl residues, in contrast, are of very recent origin

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(Yankeelov *et al.* 1966, 1968; Grossberg & Pressman 1968), but have not been tested successfully on arginyl residues of enzymes. Arginyl residues of carboxypeptidase have been examined using a 150-fold excess of diacetyl in borate buffer at pH 7.5. Peptidase activity is lost rapidly and concomitantly esterase activity increases three- to fourfold. The inhibitor, β-phenylpropionate affects this reaction only slightly, in contrast to its effect on most modifications of tyrosine. Amino acid analyses suggest that the alterations of activity are due to modification of one or two arginyl residues per mole of enzyme (Vallee & Riordan 1968). The X-ray data identify an arginyl residue as one of the binding sites for glycyl-L-tyrosine, interacting with the C-terminal carboxyl group (Lipscomb *et al.* 1968; Reeke *et al.* 1967). Our chemical data further indicate that, if an arginyl residue is modified, it does not appear to be essential to the binding of the ester, HPLA, which is still hydrolysed by the diacetyl treated enzyme (Vallee & Riordan 1968).

We have also attempted to detect functional carboxyl groups in peptidase activity as suggested by the X-ray data (Lipscomb *et al.* 1968; Reeke *et al.* 1967). In preliminary studies, coupling of carboxypeptidase with glycine methyl ester by using cyclohexylmorpholinoethyl carbodiimide was found to decrease both esterase and peptidase activities in parallel, which suggests that a carboxyl group may also participate in esterase activity. However, other carboxyl group modifying procedures can affect these two activities differentially (Vallee & Riordan 1968). This problem is under investigation.

Summarizing these chemical modifications, at least four different types of residues seem to be essential to the action of carboxypeptidase A, constituting significant progress since a mechanism was proposed. Modification of one of the two active centre tyrosyl residues can affect only esterase activity while modification of the other affects both peptidase and esterase activity. There is chemical evidence for the involvement of histidyl and arginyl residues in peptidase activity and X-ray data for arginine. Carboxylate residues may participate in both activities. There is little evidence concerning the function of other amino acid residues. The information is beginning to account for the problem initially posed, i.e. simultaneous alterations in both activities by a satisfying scheme involving all residues involved in catalysis and/or binding of both types of substrates. However, aside from the progress to be expected in understanding function from structural studies, further assistance may be anticipated from the response of environmentally sensitive, chemically modified residues to substrates and inhibitors.

Such chemically modified amino acid residues have served as useful structural probes by introducing a chromophore into the active site of the enzyme, much like the introduction of a cobalt atom already discussed. The single nitrotyrosyl residue of nitrocarboxypeptidase has an apparent pK of 6.3 compared to 7.0 for the model compound, *N*-acetyl-3-nitrotyrosine. Other tyrosyl residues of the enzymes when nitrated exhibit a pK of 6.9. The abnormally low pK of the active centre nitrotyrosyl residue could be due to features of its immediate chemical environment (Riordan, Sokolovsky & Vallee 1967*b*). The pH dependence of tyrosine nitration and the rates of tyrosyl residues in synthetic copolymers have shown that only ionized tyrosine reacts with TNM. Moreover, the ionization of a tyrosine is shifted to a lower pH if its environment contains positively charged residues, as in lysyl-tyrosine copolymers. Such data suggested that the active centre tyrosine also may have an unusually low pK due to its close proximity to a positively charged residue in the native enzyme (Riordan & Sokolovsky 1967).

The addition either of substrates or the inhibitor, β-phenylpropionate, normalizes the pK of the nitrotyrosyl residue. The resultant change in its absorption spectrum indicates that these agents alter the immediate chemical environment of this residue. It has been pointed out that

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this could be due to direct interaction of such agents with the nitrotyrosyl group or to conformational changes which may move it away from the positively charged group into a more hydrophobic environment (Riordan *et al.* 1967*b*; Riordan & Sokolovsky 1967). The interpretation of the X-ray data on carboxypeptidase is in accord with these findings (Lipscomb *et al.* 1968; Reeke *et al.* 1967).

Coupling of tyrosyl residues with *p*-azobenzenearsonate introduces a different chromophoric substitutent into the protein which serves to quantitate the reaction and to probe the chemical environment of the modified residues (Kagan 1968). Arsanilazocarboxypeptidase exhibits multiple, extrinsic ellipticity bands with peaks at 530, 430 and 328 nm.

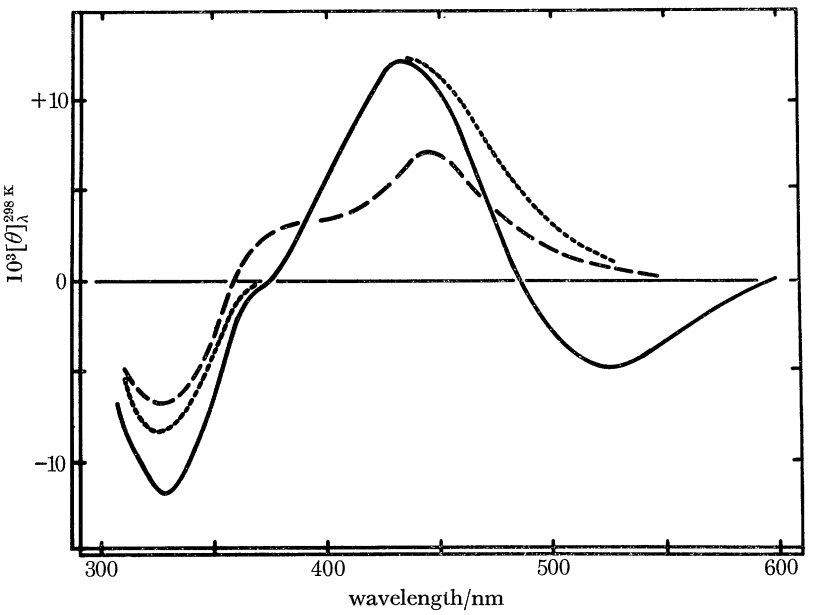

FIGURE 4. The effect of 2.5 mmol l^{-1} of *β*-phenylpropionate: (– – –) and 2.5 mmol l^{-1} of glycyl-L-tyrosine: (---) on the circular dichroism spectrum of arsanilazocarboxypeptidase. Spectra were measured in 40 mmol l^{-1} of Tris chloride 1 mol l^{-1} of NaCl, pH 7.7, 298 K.

On addition of either *β*-phenylpropionate or glycyl-L-tyrosine, the band at 530 nm disappears; changes at shorter wavelengths are also observed (figure 4). Apparently, the binding of substrates and inhibitors alter the conformation of an azotyrosyl residue.

Kinetic studies should assist in defining both the mode of action of carboxypeptidase and the basis for the changes in esterase and peptidase activity consequent to chemical modification. Over the past 25 years numerous studies have emphasized the remarkably complex kinetic behaviour of this enzyme (for detailed references see Vallee & Riordan (1968), Lipscomb *et al.* (1968), Neurath *et al.* (1968)). Various combinations of substrate and product inhibition and activation have been observed with a number of substrates and their products for the native enzyme, both in solution (Davies *et al.* 1968; Lumry, Smith & Glantz 1951; Whitaker, Menger & Bender 1966; Kaiser, Awazu & Carson 1965; McClure, Neurath & Walsh 1964) and in the crystalline state (Quiocho & Richards 1966).

Our past kinetic investigations have dealt with native, metal substituted and organically modified carboxypeptidases acting upon CGP, BGP and HPLA. The various metalloenzymes were studied to discern whether the kinetic anomalies might reside in the metal atom alone.

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This does not seem to be the case. All of the active peptidases, i.e. Mn, Co, Ni, Zn are subject to both activation and inhibition by CGP and to activation by BGP. These, as well as the Cd, Hg, and Rh enzymes catalyse the hydrolysis of HPLA, and exhibit substrate inhibition (Davies *et al.* 1968). The anomalies persist on exchanging metals for one another.

Therefore, the kinetic anomalies encountered with small substrates reflect the topological organization of the active centre and its mode of interaction with substrates. Efforts to reduce the kinetic information to a common basis have resulted in a model for substrate and product binding (Vallee 1967; Vallee *et al.* 1968).

Multiple protein–substrate interactions have been proposed in the past which differ primarily in the number of protein or substrate molecules assumed to interact (Lumry *et al.* 1951; Whitaker *et al.* 1966; Kaiser *et al.* 1965; McClure *et al.* 1964; Quiocho & Richards 1966). However, the present model (Vallee *et al.* 1968) further encompasses the dual specificity of the enzyme towards esters and peptides† and its curtailment as critical to its design. A number of different approaches have indicated that certain dipeptides differ from the ester, HPLA, in their presumable manner of binding to and hydrolysis by native carboxypeptidase. These findings have been taken into account in devising the model depicted in figure 5. Discrete sites, composed of unspecified numbers of binding loci for esters, the black symbols, and for peptides, the white symbols, are postulated. Additional pictorial representations have been published and should be inspected for details (Vallee *et al.* 1968; Vallee & Riordan 1968). The enzyme is thought to respond to the overall structural differences between certain esters and peptides and not solely to the —COO— and the —CONH— bonds. An overlap of the site for esters and peptides is proposed solely for the sake of visualization and is symbolized by the superpositioning of the black and white spheres with the adjacent grey area of the groove. While consistent with the mutual competition of ester and peptide substrates observed kinetically, the concept of overlap is not prerequisite to the model. The metal atom is omitted, consistent with the schematic intent which does not identify loci with specific chemical groups.

In the simplest case, hydrolysis of the peptide might occur only at the peptide site, the white groove; however, each type of substrate could bind in an inhibitory manner at the site for the other. Additional, catalytically unproductive complexes would be obtained when the substrate binds only to one of the loci of its active site. To observe substrate inhibition kinetically, the substrate would have to bind in more than one inhibitory manner while for non-competitive substrate inhibition to be manifested, only one such mode of binding would be required. Substrate activation would occur if the peptide were to bind at an *ester locus* (or the ester at a *peptide locus*).

While the symbols in figure 5 represent *substrate* binding, the effect of *product* binding could be visualized by considering the cubes or spheres alone. A given product would either activate or inhibit hydrolysis, depending on its site of interaction. The model predicts that a product or modifier which would activate the hydrolysis of a peptide substrate bound correctly to its site should inhibit the hydrolysis of an ester substrate bound correctly to its site. This hypothesis is consistent with results obtained using the *N*-substituted products of dipeptide and ester substrates, BG, CG, or CGGG as well as a number of related compounds (Vallee *et al.* 1968; Davies *et al.* 1968; Vallee & Riordan 1968; Davies, Auld & Vallee 1968). Figure 6 exemplifies

† A discussion of peptidase or esterase kinetics based on studies of a particular substrate need not pertain to all substrates. In such instances semantic laxity might inadvertently imply conclusions which data currently available do not allow.

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this phenomenon showing the effect of the products CG and BG on the peptide BGP and its ester analogue HPLA.

Differences in the type of inhibition observed with a series of α-keto acid inhibitors for BGP and HPLA hydrolysis have been thought to be in accord with this model (Adelman & Lacko 1968).

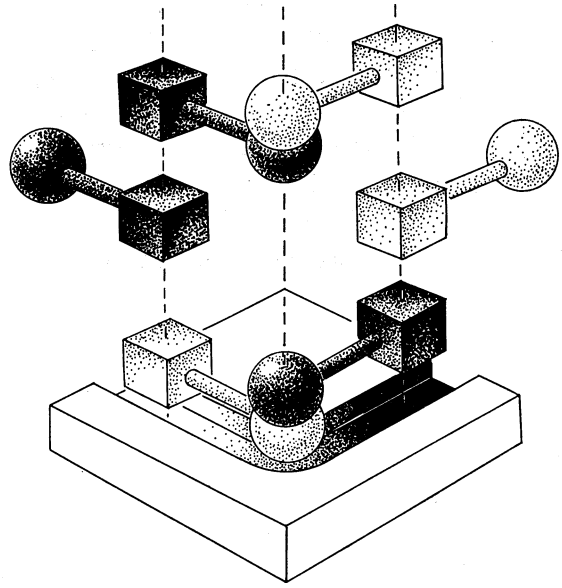

FIGURE 5. Model depicting alternative possible modes of binding of esters (black symbols) and peptides (white symbols) to carboxypeptidase A. An area of overlap, is indicated by the coincidence of the symbols and the grey shading of the groove in the centre.

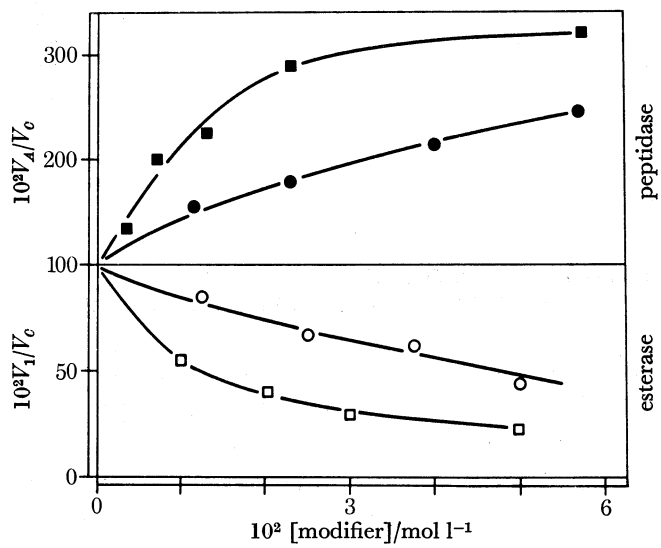

FIGURE 6. Activation of (1 mmol l^{-1}) BGP hydrolysis and inhibition of HPLA (1 mmol l^{-1}) hydrolysis as a function of the concentration of BG (●, ○) and CG (■, □). Peptidase assays were performed in 1 mol l^{-1} of NaCl, 50 mmol l^{-1} of Tris, pH 7.5 and 298 K, esterase assays in 0.2 mol l^{-1} of NaCl, 5 mmol l^{-1} of Tris, pH 7.5 and 298 K.

The characteristics of acetyl and iodocarboxypeptidase are further consistent with such a model. These enzymes are virtually inactive toward peptide substrates due, in some measure, to reduced binding of peptides as evidenced by gel filtration, isotope exchange and kinetic

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studies (Vallee 1964*b*). However, esterase activity is increased and substrate inhibition is displaced to much higher HPLA concentrations (Vallee *et al.* 1968; Davies *et al.* 1968; Vallee & Riordan 1968). Acetylation could partially or completely interfere with correct, productive peptide binding and simultaneously weaken or abolish erroneous, unproductive and inhibitory binding of the ester at the peptide site. Ester substrate inhibition would not be abolished completely, since certain modes of unproductive ester binding at the ester site could persist. The inhibition by peptides of the esterase activity of the acetylenzyme would then be viewed as unproductive binding of peptides at the ester site (Vallee *et al.* 1968).

Not all substrates evidence the kinetic anomalies encountered in the past in the study of dipeptides and depsipeptides. The hydrolysis of some oligo- and polypeptides is of this kind (Auld 1968). Thus, some substrate inhibition can still be apparent, but the substrate activation seen for BGP and CGP is no longer observed for BGGP, CGGP and CGGGP (figure 7).

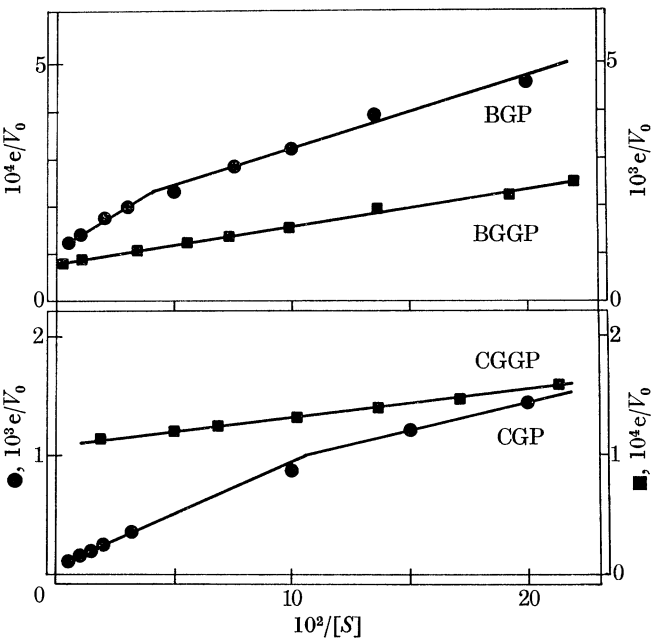

FIGURE 7. Double reciprocal plots for the hydrolysis of di- (BGP, CGP) and tri-peptides (BGGP, CGGP) performed in 1 mol l^{-1} of NaCl, 50 mmol l^{-1} of Tris, pH 7.5, 298 K.

Moreover, carbobenzoxyglycine, a known activator of dipeptides, competitively inhibits both BGGP and CGGP with a K_I of 25 mmol l^{-1} (Auld 1968). The systematic use of the L- and D-isomers of a series of alanyloligopeptides has led to the conclusion that up to five enzyme residues may be available for substrate binding, thus perhaps accounting for the kinetic ambiguities observed when small substrates are employed (Schechter & Berger 1966, 1967; Abramowitz, Schechter & Berger 1967).

The hydrolysis of peptides differs from that of esters even when both types of substrates are devoid of apparent substrate activation. Thus, β-phenylpropionate and phenylacetic acid are competitive inhibitors of hippuryl-L-mandelate (Kaiser & Carson 1965), and non-competitive inhibitors of CGGP hydrolysis (Auld 1969). Further, although the pH dependencies of V_{max}/K_m for both BGGP and acetyl mandelate (Carson & Kaiser 1966) are bell-shaped, the V_{max} against pH profile is sigmoid for the former and bell-shaped for the latter (Auld 1969).

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The peptide mechanism (figure 8) redrawn from figure 1 to allow comparison with the interpretation of X-ray data (Lipscomb *et al.* 1968; this volume, p. 177) was designed to account for all changes in esterase and peptidase activities by the modification of a *single* group involved in the catalytic step (Vallee *et al.* 1963) and has provided a challenging and productive scheme. About ten groups involved in activity including metal ligands, catalytic and binding groups have now been detected. In turn, this has prompted the development of the substrate binding model (figure 5).

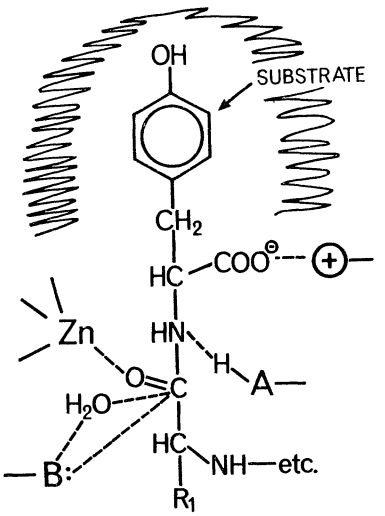

FIGURE 8. The mechanism of peptide hydrolysis is redrawn from figure 3 of Vallee *et al.* (1963) and figure 7 of Vallee (1964*a*) to coincide with the mode of presentation of Lipscomb *et al.* (1968). An *N*-substituted tyrosyl peptide is shown as the substrate. The phenolic residue of the peptide is thought to interact with a hydrophobic region of the protein, the specificity determining carboxyl group with a positively charged residue, the peptide –NH– with an acidic group, AH, and the peptide carbonyl with the catalytically essential zinc. A nucleophile, –B, initiates catalysis either directly or through an intermediate molecule of H_2O. The zinc binding site is shown to conform with Lipscomb *et al.* (1968) and Reeke *et al.* (1967).

Figure 8 shows three rather than two ligands to the zinc atom, consistent with both the comparison of the data on the metal binding sites of procarboxypeptidase and carboxypeptidase (Vallee & Riordan 1968; Piras & Vallee 1967) and the X-ray data on carboxypeptidase (Lipscomb 1968; Reeke *et al.* 1967). Their identities have been omitted owing to the chemical problems discussed, the lack of correspondence of chemical with X-ray data and pending final assignments on completion of the chemical sequence. Further, the spectral properties of cobalt carboxypeptidase and their perturbation by substrates and inhibitors have given new perspectives on the possible geometry of the metal binding site and its pertinence to catalysis (Vallee & Williams 1968*a*, *b*). Such data may also bear on the enzymic specificities induced by other metals, e.g. Cd, Hg, Rh and Pb.

While it could not be stated in our initial proposal (Vallee 1964*a*) whether or not group A or B were identical with either of the two tyrosyl residues, the nucleophile group B, postulated to initiate peptide hydrolysis, has been assigned to Glu-270 (Lipscomb 1968; Reeke *et al.* 1967). Indeed, carboxylate modification (Vallee & Riordan 1968) can alter both activities, though equivalence between chemical and structural data has not been established as yet. While a number of studies (Ginodman, Mal'tsev & Orekhovich 1966; Hall & Kaiser 1967; McClure & Neurath 1966) have tended to make the formation of an acyl enzyme intermediate less likely, a decision on the occurrence of nucleophilic versus general base catalysis still cannot be made (Vallee *et al.* 1963; Vallee 1964*a*). Kinetic data indicate that a group with a pK close to 6 and a ΔH_i between

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25 and 30 kJ mol^{-1} is involved in the catalytic step of hydrolysis of tripeptides (Auld 1969). The participation of this group directly as a nucleophile or indirectly affecting the catalytic step cannot be discerned at this time. Such uncertainties again arise from comparisons of the properties of enzymes with those of models and from the potential difficulties of equating kinetically determined pK to those of protein groups (Bruice & Schmir 1959). A histidyl residue has been implicated chemically in dipeptide hydrolysis, but its specific role is quite uncertain.

The acidic group, AH, postulated to donate its proton to the nitrogen of the C-terminal residue (Vallee *et al.* 1963; Vallee 1964*a*) has now been equated with one of the tyrosines altered by various chemical reagents (Lipscomb *et al.* 1968; Reeke *et al.* 1967). The correspondence of the sequence of an iodotyrosyl peptide isolated from iodocarboxypeptidase (Roholt & Pressman 1967) with that surrounding Tyr-248 led to the assignment of this residue to that labelled AH in this scheme. The role in peptide hydrolysis of the second tyrosyl residue, modified by several reagents, has not been defined as yet. One or both of these tyrosyl residues appear to play some role in substrate binding, but their participation in catalysis and binding respectively is undifferentiated thus far. The sensitivity of nitro and azotyrosine derivatives (Kagan 1968; Riordan *et al.* 1967*a*) to substrates and inhibitors could reflect either catalytic or binding residues or both, and the conformational changes observed for Tyr-248 on substrate binding by X-ray analysis emphasize that such differentiation of catalytic and binding roles might be specious in this instance.

The positioning of the peptide bond near the zinc atom was based on the effects of various substrates and inhibitors on binding of zinc to the apoenzyme (Coleman & Vallee 1964). In contrast, similar considerations led us to locate the carboxyl group of β-phenylpropionate at the zinc atom (Coleman & Vallee 1964) as since confirmed by X-ray studies with β-(p-iodo)-phenylpropionate (Lipscomb *et al.* 1968) and by n.m.r. studies on manganese carboxypeptidase with β-phenylpropionate (Shulman *et al.* 1966).

Locating the carbonyl group of the peptide bond near the zinc atom necessitated the incorporation into the scheme of a positively charged residue to interact with the C-terminal carboxyl group of the substrate. For glycyl-L-tyrosine this binding group has now been identified as Arg-145 (Reeke *et al.* 1967). Chemical studies have indicated the functional importance of one or two arginyl residues for peptidase activity (Vallee & Riordan 1968).

Thermodynamic and kinetic data suggesting differences in peptide and ester binding have led to the substrate binding model presented (Vallee 1967; Vallee *et al.* 1968). There is much less experimental information on the behaviour of ester than of peptide substrates by thermodynamic, kinetic or structural criteria, and present mechanistic considerations of ester hydrolysis remain largely conjectural. However, binding studies with acylamino acids and esters show that they do not interact with the active centre as do dipeptides (Coleman & Vallee 1962*a*, *b*). In this regard their behaviour resembles that of β-phenylpropionate (Coleman & Vallee 1964). Further, competitive inhibitors of the hydrolysis of esters have been found to be non-competitive inhibitors of tripeptide hydrolysis (Auld 1969). The possibility that the location of the ester bond on one hand and the carboxyl group on the other relative to the zinc atom differs from that of dipeptides cannot be dismissed without experimental examination. Arginyl modification, while diminishing CGP hydrolysis actually increases HPLA hydrolysis (Vallee & Riordan 1968) suggesting that the arginyl group involved in glycyl-L-tyrosine and, by inference, CGP binding, may not serve the same function in HPLA hydrolysis.

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Summary

This discussion has traced the development of current knowledge and ideas on functional characteristics of carboxypeptidase A. This metalloenzyme has presented unusual opportunities for the exploration of its enzyme action by virtue of its functional responses to metal substitution and organic modifications. Perturbations of both organic and inorganic chromophores by substrates and inhibitors provide new approaches. The X-ray examination of this system affords the first opportunity for the comparison of the conclusions derived from functional and structural studies of a metalloenzyme. While pointing to the limitations in the use of models for the understanding of the unique characteristics of active sites of enzymes, the results of chemical and X-ray analysis are proving to be remarkably complementary.

This work was supported by Grant-in-aid GM 15003 from the National Institutes of Health of the Department of Health, Education and Welfare. D. S. Auld is a Fellow of the American Cancer Society, and S. A. Latt is a postdoctoral fellow of the National Institutes of Health, Division of Arthritis and Metabolic Diseases.

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