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INTRINSIC CATALYTIC ACTIVITY OF PROCARBOXYPEPTIDASE A

A KINETIC STUDY USING FLUORINE ANALOGUES

PATRICIA C. PLESE * and W. DAVID BEHNKE **

Department of Chemistry, University of South Carolina, Columbia, S.C. 29208 and Department of Biological Chemistry, University of Cincinnati College of Medicine, 231 Bethesda Avenue, Cincinnati, Ohio 45267 (U.S.A.)

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Summary

Bovine procarboxypeptidase A displays substantial catalytic activty toward halogenated acyl-amino acids, the most active of which is trifluoroacetyl-L-phenylalanine (TFAc-L-Phe). Though this activity is not as great as for the native enzyme, it is quite substantial and far beyond the range of adventitious activation. Both DL-benzylsuccinate and β-phenylpropionate inhibit zymogen hydrolysis of TFAc-L-Phe, the former with a K_I of 4.1 μM and the latter, 900 μM (a value much higher than the corresponding enzyme).

Apo procarboxypeptidase A will also hydrolyze TFAc-L-Phe, presumably the polarization of the carbonyl carbon being accomplished by the fluorine atoms in the absence of a specific metal ion. That this is not entirely the metal ion function is indicated by the fact that rate enhancements follow the order manganese procarboxypeptidase A ≈ zinc procarboxypeptidase > apo-procarboxypeptidase. The results indicate considerable similarities for the zymogen-enzyme pair in terms of catalytic groups, pH dependence, specificity and the nature of their transition state binding sites. Some changes in the substrate or inhibitor binding sites are noted.

Introduction

The formation of the active sites of the proteolytic enzymes is generally thought to occur through induced alterations in the zymogen conformations

* In partial fulfillment for a Masters Degree, University of South Carolina, Columbia, S.C., U.S.A.

** To whom correspondence should be addressed.

Abbreviations: procarboxypeptidase A-S6, the enzyme having a sedimentation rate, S, of 6, a trimer; procarboxypeptidase A-S5, the enzyme having a sedimentation rate, S, of 5, a dimer.

which are produced by the cleavage of the primary structure [1]. In recent years, however, it has become increasingly evident that the features of the active sites of proteolytic enzymes pre-exist in the zymogens. Numerous studies with pepsinogen, trypsinogen, chymotrypsinogen, and procarboxypeptidase have indicated the pre-existence of a catalytic site; in fact, some of these zymogens have been shown to possess an inherent catalytic activity [2,3]. Investigators of the pepsinogen to pepsin conversion have shown through kinetic studies that the activation is self-catalyzed and that the intramolecular cleavage of the activation peptide probably takes place at the same active site that is present in pepsin [4,5].

Catalytic activity was reported for procarboxypeptidase A as early as 1963 [6]. Since then there have been numerous studies reporting procarboxypeptidase A activity. ^{65}Zn exchange in procarboxypeptidase A-S6, which is retarded by the addition of substrates, substrate analogs, and inhibitors similar to results demonstrated with the enzyme, suggests the presence of features of the binding site in the zymogen [7,8].

Lacko and Neurath [9], using a monomeric zymogen of carboxypeptidase A isolated from the spiny Pacific dogfish (*Squalus acanthias*), showed the presence of catalytic activity and determined the kinetic parameters, k_{cat} and K_m, for several substrates. Hass and Neurath [10] and later, Uren et al. [11, 12] demonstrated by chemical modification that catalytically functional groups, zinc, tyrosine and glutamic acid (corresponding to Tyr-248 and Glu-270 of the enzyme), were pre-existent in procarboxypeptidase A-S5.

The circular dichroic and magnetic circular dichroic spectra of cobalt procarboxypeptidase A are distinctive and essentially identical to similar spectra for the cobalt-substituted enzyme [13]. These spectra, which are characterized by low symmetry and irregular geometry for the metal atom, are thought to reflect catalytic potential for a metalloprotein [14—16]. This led to the development of a new class of substrates for the Co-zymogen, e.g. the haloacylated amino acids [13] which were later found to be hydrolyzed by zinc procarboxypeptidase A [17]. These substrates are minimal in size and complexity and meet the specificity requirements of the enzyme.

This study is a characterization of one of the halogenated substrates, trifluoroacetyl-L-phenylalanine. The use of this halogenated derivative provides a new channel for the study of zymogen activation using ^{19}F NMR techniques. In addition, catalysis by both apo-carboxypeptidase A and procarboxypeptidase A can be demonstrated in the absence of metal ions; a mechanistic explanation is provided.

Materials and Methods

Zinc carboxypeptidase, (twice crystallized) was purchased from Worthington Biochemical Corp. An aliquot of the crystalline suspension was suspended in 1 ml of cold, glass-distilled water. The solution was centrifuged for 5 min at 3400 rev./min the supernatant drawn off, and the pellet resuspended in 1 ml of cold, glass-distilled water. The centrifugation was repeated and the pellet was dissolved in a 2 M NaCl 0.05 M Tris, buffer, pH 7.5. For enzymatic assay of zinc carboxypeptidase A, a stock solution of 1 mg/ml was routinely prepared.

Zinc procarboxypeptidase was prepared using the procedure of Brown et al. [18] as modified by Behnke et al. [19].

Because of the ease of solubilizing the zymogen, the solvent chosen for the stock solution (usually $1 \cdot 10^{-4}$ M zymogen) was the buffer system for the assay.

Protein concentrations were determined using a Gilford Model 240 spectrophotometer from the absorbance at 280 nm where $\epsilon^{0.1\%}_{280\,nm} = 1.88$ [20]. The protein molecular weights used were 33 800 for the enzyme [21] and 88 000 for the zymogen [6].

Preparation of apo-carboxypeptidase A and manganese carboxypeptidase A. An aliquot of the crystalline zinc carboxypeptidase A suspension was washed twice with cold, glass-distilled water as in the treatment of zinc carboxypeptidase A. The pellet was then suspended in 3 ml of 0.05 M Tris, $2 \cdot 10^{-3}$ M, 1,10-phenanthroline (reagent grade), pH 7.5; the tube sealed with parafilm; and the suspension chilled for 5 min in an ice bath. The suspension was then centrifuged at 3400 rev./min for 5 min and the supernatant drawn off with a metal-free disposable pipet. This process was repeated five times after which the pellet was then suspended in 3 ml of metal-free 0.05 M Tris, pH 7.5, and the procedure repeated. After this second washing the apoenzyme was stored as a pellet under 0.05 M Tris, pH 7.5. Manganese carboxypeptidase A was prepared by dissolving the apoenzyme crystals in a 3 M NaCl, 0.05 M Tris buffer, pH 7.5, measuring the protein concentration and adding spectroscopically pure $MnSO_4$ (Johnson, Mathey Chemicals Ltd.) in equimolar concentration from a 0.1 M aqueous stock solution.

Preparation of apo-procarboxypeptidase A and manganese procarboxypeptidase A. The procedure used was a modification of the preparation as described by Piras and Vallee [8]. The lyophilized protein was dissolved in a 0.05 M Tris, 1.0 M NaCl, pH 7.5, solution. The protein solution was dialyzed (ten changes) against a 100-fold volume excess of a $2 \cdot 10^{-3}$ M 1,10-phenanthroline, 0.05 M Tris, pH 7.5, solution at 4°C. After the tenth dialysis, the apoprotein was dialyzed against an extracted 0.05 M Tris, pH 7.5, 1.0 M NaCl solution for four buffer changes over a period of 10 h.

The apozymogen was stored at 0°C in a metal-free container. To insure the the presence of manganese procarboxypeptidase A the assays were run with 10^{-4} M excess $MnSO_4$ as part of the reaction mixture.

Metal-free buffers and reagent solutions. All buffers and reagents were prepared from reagent grade chemicals. The solutions were extracted with 0.001% dithizone in carbon tetrachloride to remove divalent metal ions. Precautions to avoid metal ion contamination were those employed by Thiers [22]. The extraction was repeated until no visible color change was observed in the dithizone phase and the residual dithizone is removed by continued extraction with carbon tetrachloride.

Preparation of substrates and inhibitors. Trifluoroacetyl-L- and -D-phenylalanine (TFAc-L-Phe and TFAc-D-Phe) were prepared by the treatment of D- or L-phenylalanine (Sigma Chemical Co.) with anhydrous trifluoroacetic anhydride at 0°C. Crystallization occurred spontaneously on addition of water and the derivative was then twice recrystallized from a water/ethanol mixture.

β-Phenylpropionate was purchased from Fisher Chemical Co. and was recrystallized from light petroleum.

DL-benzylsuccinic acid was obtained from Dr. R. Wolfenden, University of North Carolina Medical School.

Activity measurements. For all studies, except the pH profiles, the rate measurements were determined in a 1.0 M NaCl, 0.05 M Tris buffer, pH 7.5. The temperature was controlled at 25°C with a Haake E52 constant temperature bath. pH determinations were made with a Radiometer pHM 63 digital pH meter.

The assay system used was that developed by Auld and Vallee [23] using the ninhydrin reaction and a Technicon Autoanalyzer (Technicon Instruments Corp., Tarrytown, N.Y.). Assay mixtures were prepared to which either 0.2 or 0.5 ml of enzyme or zymogen solution was added to initiate the reaction. Sample aliquots (0.2 or 0.5 ml) were withdrawn at fixed time intervals and the reaction was quenched with a 1.0 M sodium citrate buffer, pH 5.0.

Standard curves were prepared using the amino acid product of the hydrolysis (L-phenylalanine).

The assay mixture consisted of 0.01 M TFAc-L-Phe, 0.05 M buffer, 1.0 M NaCl, and either $1 \cdot 10^{-9}$ M enzyme or $2 \cdot 10^{-8}$ zymogen. For the alkaline region, 0.005 M TFAc-L-Phe was used because of high background, presumably caused by base-catalyzed substrate hydrolysis. At pH values greater than 8.5 controls were run to correct for background hydrolysis. The pH was determined before and after the assay using the remainder of the reaction mixture. At alkaline pH values, pH corrections were made for the high sodium content (1.0 M NaCl) in the assay mixture [24].

Results

The turnover rates for the hydrolysis of the halogenated substrate, trifluoroacetyl-L-phenylalanine (TFAc-L-Phe), are large for both zinc carboxypeptidase A and zinc procarboxypeptidase A with V values of $16.6 \cdot 10^3$ min^{-1} and $1.8 \cdot 10^3$ min^{-1}, respectively. Lineweaver-Burk plots (not shown) are linear over a 10-fold concentration range. This differs from the results exhibited by other small molecule substrates such as carbobenzoxylyl-L-phenylalanine which deviates markedly from linearity. The K_m values are 1.49 mM for the enzyme and 2.6 mM for the zymogen.

The variation of log (k_{cat}/K_m) as a function of pH for the Zn-zymogen and enzyme are very similar, data not shown. The apparent pK values for the ionizing groups have been calculated [25,26] for each protein. For the enzyme in the acid range, a pK of 6.5 is indicated, while for the zymogen this value is 6.3.

Similar treatment of a log k as a function of pH curve yields apparent pK values of 6.3 and 8.8 for ionizable groups for both zinc carboxypeptidase A and zinc procarboxypeptidase A which approximates the results for the acid region calculated previously [3].

Dixon plots indicate that trifluoroacetyl-L-phenylalanine (TFAc-D-Phe) is a competitive inhibitor of both zinc carboxypeptidase A and zinc procarboxypeptidase A with K_i values which are quite similar, 6.1 and 6.5 mM, respectively (Figs. 1A and 1B).

DL-Benzylsuccinic acid (DL-BSA) was shown by Byers and Wolfenden [27]

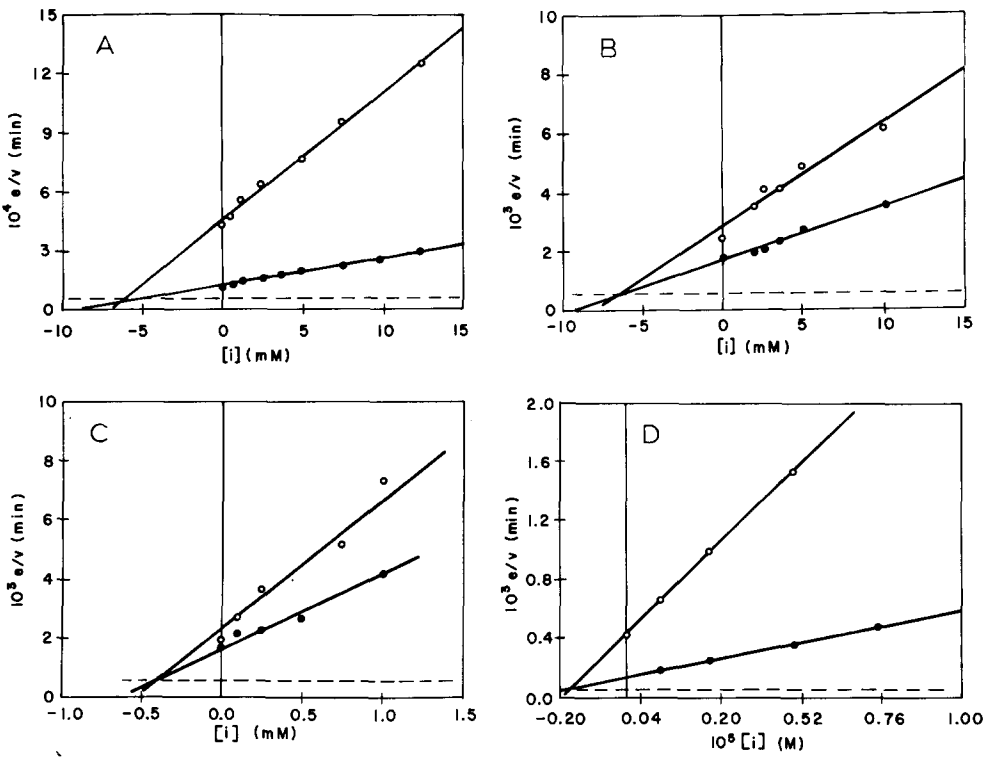

Fig. 1(A) Dixon plot of the inhibition of zinc carboxypeptidase A hydrolysis of TFAc-L-Phe by TFAc-D-Phe. Experimental conditions: 0.05 M Tris, 1.0 M NaCl, pH 7.5, 25°C. (○———○), $2.5 \cdot 10^{-4}$ M TFAc-L-Phe; ●———●, $1.22 \cdot 10^{-3}$ M TFAc-L-Phe. $1/V$ (- - - - -) determined from Lineweaver-Burk plot. (B) Dixon plot of the inhibition of zinc procarboxypeptidase A hydrolysis of TFAc-L-Phe by TFAc-D-Phe. Experimental conditons: 0.05 M Tris, 1.0 M NaCl, pH 7.5, 25°C, $2.76 \cdot 10^{-8}$ M zinc procarboxypeptidase A. ○———○, $1.0 \cdot 10^{-3}$ M TFAc-L-Phe; ●———●, $2.5 \cdot 10^{-3}$ M TFAc-L-Phe. $1/V$ (- - - - -) determined from Lineweaver-Burk plot. (C) Dixon plot of the inhibition of zinc procarboxypeptidease A hydrolysis of TFAc-L-Phe by DL-BSA. Experimental conditions: 0.05 M Tris, 1.0 M NaCl, pH 7.5, 25°C, $3.14 \cdot 10^{-8}$ M zinc procarboxypeptidase A. ○———○, $2.5 \cdot 10^{-3}$ M TFAc-L-Phe; ●———●, $5.0 \cdot 10^{-3}$ M TFAc-L-Phe. $1/V$ (- - - - -) determined from Lineweaver-Burk plot. (D) Dixon plot of the inhibition of zinc carboxypeptidase A hydrolysis of TFAc-L-Phe by DL-BSA. Experimental conditions: 0.05 M Tris, 1.0 M NaCl, pH 7.5, 25°C, $5.83 \cdot 10^{-9}$ M zinc carboxypeptidase A. ○———○, $1.0 \cdot 10^{-3}$ M TFAc-L-Phe; ●———●, $5.0 \cdot 10^{-3}$ M TFAc-L-Phe. $1/V$ (- - - - -) determined from Lineweaver-Burk plot.

to be a potent inhibitor of carboxypeptidase with a binding constant several orders of magnitude tighter than other known inhibitors. DL-BSA was found to be a powerful inhibitor of TFAc-L-Phe hydrolysis by both the zymogen and enzyme yielding K_i values of 4.1 and 1.86 μM, respectively (Figs. 1C and 1D, Table I).

A Lineweaver-Burk plot of the hydrolysis of TFAc-L-Phe by manganese carboxypeptidase A yielded a V of $103 \cdot 10^3$ min^{-1}, (Fig. 2A), a value nearly sixty times greater than that for the Zn-zymogen which is $1.88 \cdot 10^3$ min^{-1} (Fig. 2B). The K_m values are 10 and 4.3 mM for the enzyme and zymogen, respectively.

DL-BSA was also found to inhibit the Mn-enzyme and zymogen competitively with the same high binding affinity as observed above (vide supra). The K_i values for these metal-substituted proteins are 2 μM for the enzyme and 4 μM for the zymogen (Figs. 2C and 2D).

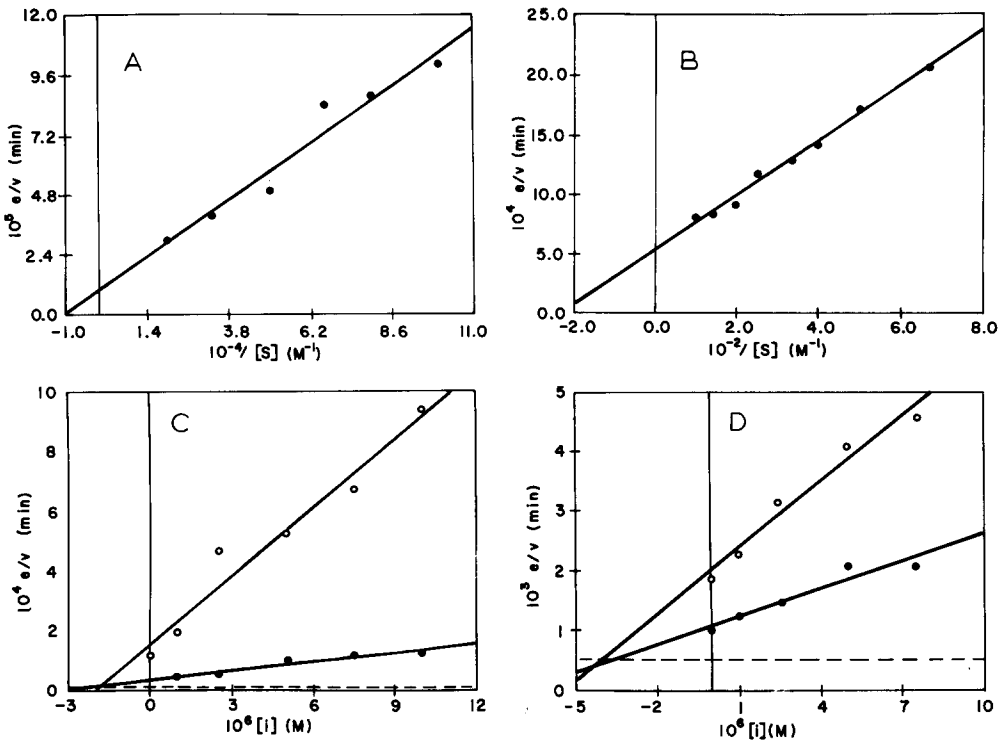

Fig. 2. (A) Lineweaver-Burk plot of the hydrolysis of TFAc-L-Phe by manganese carboxypeptidase A. Experimental conditions: 0.05 M Tris, 1.0 M NaCl, pH 7.5, 25°C, $1.42 \cdot 10^{-8}$ M manganese carboxypeptidase A. (B) Lineweaver-Burk plot of the hydrolysis of TFAc-L-Phe by manganese procarboxypeptidase A. Experimental conditions: 0.05 M Tris, 1.0 M NaCl, pH 7.5, 25°C, $2.48 \cdot 10^{-8}$ M manganese procarboxypeptidase A. (C) Dixon plot of the inhibition of manganese carboxypeptidase A hydrolysis of TFAc-L-Phe by DL-BSA. Experimental conditions: 0.05 M Tris, 1.0 M NaCl, pH 7.5, 25°C, $1.42 \cdot 10^{-8}$ M manganese carboxypeptidase A. ○———○, $1.0 \cdot 10^{-3}$ M TFAc-L-Phe; ●———●, $5.0 \cdot 10^{-3}$ M TFAc-L-Phe. $1/V$ (- - - - -) determined from Lineweaver-Burk plot. (D) Dixon plot of the inhibition of manganese procarboxypeptidase A hydrolysis of TFAc-L-Phe by DL-BSA. Experimental conditions: 0.05 M Tris, 1.0 M NaCl, pH 7.5, 25°C $2.48 \cdot 10^{-8}$ M manganese procarboxypeptidase A. ○———○, $2.5 \cdot 10^{-3}$ M TFAc-L-Phe, ●———●, $5.0 \cdot 10^{-3}$ TFAc-L-Phe. $1/V$ (- - - - -) determined from Lineweaver-Burk plot.

β-Phenylpropionate, a competitive inhibitor of zinc carboxypeptidase A, was found to be competitive inhibitor of TFAc-L-Phe hydrolysis by both manganese procarboxypeptidase A and manganese carboxypeptidase A. The binding

TABLE I

Enzyme	V (min^{-1})	K_m (mM)	K_i		
			DL-BSA (μm)	TFAc-D-Phe (mM)	β-Phenyl-propionate (mM)
Zinc procarboxypeptidase A	$1.8 \cdot 10^3$	2.6	4.1	6.5	—
Zinc carboxypeptidase A	$16.6 \cdot 10^3$	1.49	1.8	6.1	—
Manganese procarboxypeptidase A	$1.9 \cdot 10^3$	4.3	4.0	9.3	0.9
Manganese carboxypeptidase A	$103 \cdot 10^3$	10	2.0	—	0.04

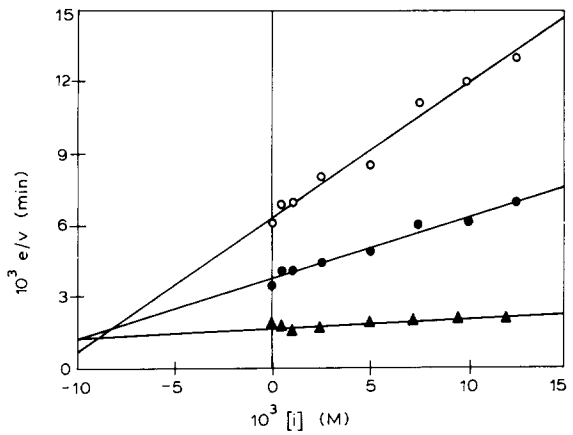

Fig. 3. Dixon plot of the inhibition of apo-procarboxypeptidase A hydrolysis of TFAc-L-Phe by TFAc-D-Phe. Experimental conditions: 0.05 M Tris, 1.0 M NaCl, pH 7.5, 25°C, $2.83 \cdot 10^{-8}$ M apo-procarboxypeptidase A. ○———○, $0.5 \cdot 10^{-3}$ M TFAc-L-Phe; ●———●, $1.0 \cdot 10^{-3}$ M TFAc-L-Phe. ▲———▲, $5.0 \cdot 10^{-3}$ TFAc-L-Phe.

constant for the enzyme is an order of magnitude greater than that of the zymogen, $4.2 \cdot 10^{-5}$ and $9 \cdot 10^{-4}$ M, respectively (unpublished data). Unexpectedly, apo-procarboxypeptidase A and apo-carboxypeptidase A were found to possess catalytic activity towards TFAc-L-Phe, the former with a K_{cat} of 670 min^{-1}. Furthermore, the apo-zymogen is competitively inhibited by TFAc-D-Phe, yielding a K_i of 9.0 mM (Fig. 3).

Table I summarizes these kinetic parameters for the native proteins and their manganese derivatives.

Discussion

The presence of identical CD spectra for cobalt procarboxypeptidase A and cobalt carboxypeptidase A suggested that prior to activation, the zymogen possessed catalytic activity [13]. The catalysis of TFAc-L-Phe by zinc and manganese procarboxypeptidase is quite substantial and, therefore, allows the determination of kinetic parameters.

The Zn- and Mn-zymogens are shown to have lower catalytic activity than their activated forms (Table I), and this is apparent by comparing the k_{cat} values. The fact that K_m values differ at all for the proteins suggests the presence of an incomplete binding site in the zymogen. This conclusion is further supported by inhibition data. K_i values are approx. 2-fold tighter for the enzyme in most of the inhibitors tested (Table I). Noteworthy are the results obtained for the transition state analogue, DL-benzylsuccinic acid (DL-BSA) in which the apparent binding constant for this inhibitor is several orders of magnitude above that of other inhibitors or for the substrate itself. DL-BSA is thought to be more effective inhibitor of carboxypeptidase because of its structural resemblance to the products of the carboxypeptidase reaction [27]. The zymogen also displays a very high affinity for this inhibitor implying the presence of such a binding site in the zymogen.

The largest difference noted in binding is the inhibition of the Mn-proteins by β-phenylpropionate. The binding constant for the zymogen is over 20-fold weaker than that for the enzyme. It has been shown that β-phenylpropionate has multiple binding loci on the enzyme [28—30]. The weaker binding by the zymogen may be a reflection of a distortion or absence of a high affinity binding locus.

One exception to the previous binding results is the inhibition of the Zn-zymogen and enzyme by TFAc-D-Phe for which the K_i values are essentially the same (Table I). This equivalence of K_i values implies that the binding elements for this inhibitor are complete in the zymogen and may indeed be the same as that for the enzyme.

Since it has been shown that apo-carboxypeptidase has no catalytic activity towards conventional substrates, the high activity of the apoenzyme, zymogen pair was unexpected. An explanation of these results might be the following: the carbonyl oxygen of the peptide bond of the substrate is thought to interact with the zinc of the active site of carboxypeptidase [31], thus polarizing the carbonyl group. Then then facilitates the nucleophilic attack of Glu-270 or H_2O. This role appears to be partially circumvented by the fluorine atoms in the TFAc-L-Phe substrate, presumably by inductive effects. The competitive inhibition by TFAc-D-Phe implies also the presence of binding sites in the apozymogen, although weaker than that of both zinc procarboxypeptidase A and zinc carboxypeptidase A. This suggests some participation of zinc in the binding of the inhibitor as well. Outside of this, all other specificity requirements must be met. Virtually no activity is observed with any derivative of the enzyme or zymogen against TFAc-L-Phe-NH_2, TFAc-D-Phe or TFAc-Gly (unpublished results), all consistent with the specificity requirements of the enzyme.

The catalytically functional groups in the two proteins appear to be similar. The pH dependence of log k and log (k_{cat}/K_m) for the hydrolysis of TFAc-L-Phe by both the Zn-zymogen and enzyme indicate the presence of similar ionizing groups affecting catalysis. Normalization of the pH vs. log (k or k_{cat}/K_m) rate profiles shows only slight deviations in the acid region [3].

Thus, the potential for catalytic activity of procarboxypeptidase appears to be realized on activation by the completion of elements of the binding site(s) and to depend less on completion of transition state elements or functional groups in the catalytic site.

References

1 Neurath, H. (1964) Fed. Proc. 23, 1—7
2 Kassell, B. and Kay, J. (1973) Science 180, 1022—1027
3 Canonici, P. and Behnke, W.D. (1974) Biochem. Biophys. Res. Commun. 56, 575—579
4 Bustin, M. and Conway-Jacobs, A. (1971) J. Biol. Chem. 246, 615—620
5 Al-Janabi, J., Hartsuck, J.A. and Tang, J. (1972) J. Biol. Chem. 247, 4628—4632
6 Yamasaki, M., Brown, J.R., Cox, D.J., Greenshields, R.N., Wade, R.D. and Neurath, H. (1963) Biochemistry 2, 859—866
7 Piras, R. and Vallee, B.L. (1966) Fed. Proc. 25, 1234
8 Piras, R. and Vallee, B.L. (1967) Biochemistry 6, 348—357
9 Lacko, A.G. and Neurath, H. (1970) Biochemistry 9, 4680—4690
10 Hass, M. and Neurath, H. (1971) Biochemistry 10, 3535—3540
11 Uren, J.R., Neurath, H. and Walsh, K.A. (1972) Fed. Proc. 31, 3989

12 Uren, J.R. and Neurath, H. (1974) Biochemistry 13, 3512—3520
13 Behnke, W.D. and Vallee, B.L. (1972) Proc. Natl. Acad. Sci. U.S. 69, 2442—2445
14 Vallee, B.L. and Williams, R.J.P. (1968) Proc. Natl. Acad. Sci. U.S. 59, 498—505
15 Williams, R.J.P. (1971) Cold Spring Harbor Symp. Quant. Biol. 36, 53—62
16 Vallee, B.L. (1973) Adv. Exp. Med. Biol. 40, 1—12
17 Bazzone, T.J. (1974) Fed. Proc. 33, 1722
18 Brown, J.R., Yamasaki, M. and Neurath, H. (1963) Biochemistry 2, 877—886
19 Behnke, W.D., Wade, R.D. and Neurath, H. (1970) Biochemistry 9, 4179—4188
20 Bargetzi, J.P., Sampath Kumar, K.S.V., Cox, D.J., Walsh, K.A. and Neurath, H. (1963) Biochemistry 2, 1468—1474
21 Bradshaw, R.A., Ericsson, L.H., Walsh, K.A. and Neurath, H. (1969) Proc. Natl. Acad. Sci. U.S. 63, 1389—1394
22 Thiers, R.E. (1957) Methods Biochem. Anal. 5, 273—335
23 Auld, D.S. and Vallee, B.L. (1970) Biochemistry 9, 602—609
24 Dole, M. (1947) The Glass Electrode, appendix V, John Wiley and Sons, Inc., New York
25 Dixon, M. (1953) Biochem. J. 55, 161—170
26 Auld, D.S. and Vallee, B.L. (1970) Biochemistry 9, 4352—4359
27 Byers, L.D. and Wolfenden, R. (1972) J. Biol. Chem. 247, 606—608
28 Simpson, R.T., Riordan, J.F. and Vallee, B.L. (1963) Biochemistry 2, 616—622
29 Bethune, H.L. (1965) Biochemistry 4, 2698—2704
30 Behnke, W.D. and Vallee, B.L. (1971) Biochem. Biophys. Res. Commun. 43, 760—765
31 Lipscomb, W.N. (1973) Proc. Natl. Acad. Sci. U.S. 70, 3797—3801