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Carboxypeptidase Inhibitor from Potatoes. Interaction with Derivatives of Carboxypeptidase A†

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ABSTRACT: The mechanism of action of a carboxypeptidase inhibitor from potatoes has been probed by studying its interaction with derivatives of carboxypeptidase A containing modified residues at the active site. Arsanilazocarboxypeptidase A, a derivative containing a chromophore attached to tyrosine 248, exhibits a circular dichroism spectrum which is sensitive to the presence of ligands at the active site (Kagan, H. M., and Vallee, B. L. (1969), *Biochemistry 8,* 4223). Since the spectral change attending binding of the carboxypeptidase inhibitor to arsanilazocarboxypeptidase A is similar to that produced by small substrates and inhibitors, the enzyme-inhibitor interaction also involves the enzyme active site. Catalytic activity is not required for inhibitor binding. Complexes of the inhibitor with apocarboxypeptidase A and carboxypeptidase A which was inactivated by treatment with the affinity label, *N*-bromoacetyl-*N*-methyl-L-phenylalanine, are demonstrated by gel filtration experiments. Moreover, competitive binding studies reveal that the latter derivative, in which the binding pocket is presumably blocked by reagent, binds inhibitor nearly as strongly as does the native enzyme, the differences in free energy of association being only 0.4 kcal/mol of a total binding energy of −11 kcal/mol. A model is proposed to account for both the tight binding of inhibitor to the *N*-bromoacetyl-*N*-methyl-L-phenylalanine derivative and the involvement of the active site of arsanilazocarboxypeptidase A. It is suggested that the inhibitor fits into a shallow depression at the active site of the enzyme but does not penetrate into the binding pocket.

In contrast to the widespread distribution of inhibitors of the "serine" proteases, inhibitors of carboxypeptidases have only been found in potatoes (Ryan, 1971) and *Ascaris lumbricoides* (Homandberg and Peanasky, 1974). The inhibitor from potatoes is a mixture of polypeptides of 38 and 39 amino acid residues. It exhibits specificity toward both carboxypeptidases A and B with inhibition constants, K_I, of 4.2×10^{-9} and 1×10^{-8} M, respectively. An earlier report (Hass et al., 1976) describes the effects of chemical modifications of the inhibitor on binding to carboxypeptidases A and B, providing evidence that these enzymes utilize the same inhibitor binding site which has been found to be the carboxyl-terminal region.

This report describes the binding of carboxypeptidase inhibitor to derivatives of carboxypeptidase A containing modified active site structures and specifically addresses the following questions: (1) Does the binding of carboxypeptidase inhibitor occur at or near the enzyme active site? (2) Is catalytic activity required for binding the carboxypeptidase inhibitor? and (3) Does interaction of carboxypeptidase inhibitor in the enzyme binding pocket contribute to the stability of the complex? These studies clarify the mechanism of action of carboxypeptidase inhibitor and provide a basis of comparison with well-documented "serine" protease-proteinase inhibitor systems.

Experimental Section

Materials. The carboxypeptidase inhibitor was prepared according to Ryan et al. (1974) with the minor modification of substituting Sephadex G-50F (equilibrated with water) and SE-cellulose (Gallard Schlesinger) for Sephadex G-75 and phosphocellulose, respectively.

A radioactive derivative was prepared by treating inhibitor (4 mg) with 10.2 mg of [^{14}C]acetic anhydride (0.1 mCi; New England Nuclear) for 30 min in an ice-salt mixture (Riordan

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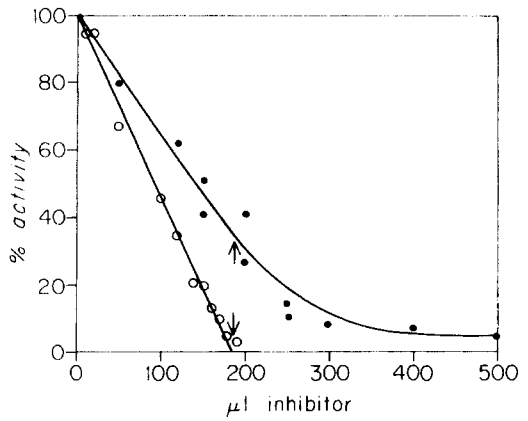

FIGURE 1: Titration of carboxypeptidase A with the carboxypeptidase inhibitor. Carboxypeptidase A (0.97 μM final concentration) was titrated with inhibitor (16 μM) using chloroacetyl-L-tyrosine as substrate (○). Carboxypeptidase A (97 nM final concentration) was titrated with inhibitor (1.6 μM) using hippuryl-L-phenylalanine as substrate (●). Stoichiometric amounts of enzyme and inhibitor in each case are indicated by the arrows.

and Vallee, 1972). The reaction was carried out in 2 ml of 0.1 M Hepes[1], which was maintained at pH 7.6 with 1.0 M NaOH. The reaction was terminated by desalting on a 1.6 × 70 cm column of Sephadex G-25 SF equilibrated in 0.01 M ammonium bicarbonate, pH 7.5.

Carboxypeptidase A was obtained from Worthington Biochemical Corp. The arsanilazo derivative was prepared by the method of Johansen and Vallee (1971). No precautions were taken against adventitious metal ions. In this work, only 0.7 mol of arsanilazo group was incorporated per mol of enzyme as measured spectrophotometrically (Tabachnick and Sobotka, 1960; Johansen et al., 1972).

Apocarboxypeptidase A was prepared by passing the enzyme through a 1.6 × 65 cm column of Sephadex G-50 F equilibrated with 0.1 mM 1,10-phenanthroline in 1 M NaCl, 50 mM Tris-HCl, pH 7.5. Metal-free inhibitor was prepared in the same manner. The removal of zinc from enzyme was successful as judged by the lack of activity of the apoenzyme (less than 5% of that of the holoenzyme) in metal-free buffers prepared by extraction with dithizone. The addition of 100 μl of 0.1 mM $ZnCl_2$ to the apoenzyme in the assay mixture fully restored activity.

BrAc(Me)Phe was prepared as described previously (Hass and Neurath, 1971a). A derivative of carboxypeptidase containing this affinity label was prepared from CPA_β^{Val} (Reeck et al., 1971; Pétra et al., 1971) by incubation with 5 mM BrAc(Me)Phe for 24 h at 24 °C in 1 M NaCl, 50 mM Tris-HCl, pH 7.5. Under these conditions, CPA_β^{Val} is completely inactivated by exclusive modification of glutamic acid 270 (Hass and Neurath, 1971a,b).

Analytical Procedures. Radioactivity measurements were performed with a Packard Tri-Carb liquid scintillation counter, Model 3003. Aqueous samples were added to 10 ml of toluene–Triton X-100 (2:1, v/v) containing 2.67 g/l. 2,5-diphenyloxazole and 0.067 g/l. 1,4-bis[2-(5-phenyloxazolyl)]benzene.

Amino acid analyses were performed on a Durrum D500 amino acid analyzer. Samples were hydrolyzed for 18–24 h prior to analysis in 6 N HCl at 110 °C evacuated from a nitrogen atmosphere (Moore and Stein, 1963).

Protein Concentrations. The concentrations of native carboxypeptidase A and its Ac(Me)Phe derivative were determined spectrophotometrically assuming an ϵ_{278} value of $6.42 \times 10^4\ M^{-1}\ cm^{-1}$ (Bargetzi et al., 1963). Amino acid analysis of appropriate aliquots of stock solutions was employed to estimate the concentrations of arsanilazocarboxypeptidase A and Ac(Me)Phe·CPA.

The concentration of carboxypeptidase inhibitor was determined based on ϵ_{280} $1.15 \times 10^4\ M^{-1}\ cm^{-1}$ calculated from the amino acid sequence (Hass et al., 1975). This method agreed within 5% with inhibitor concentrations calculated by titration with enzyme and by amino acid analysis.

Enzyme Assays. Titration of Carboxypeptidase A with Inhibitor. Carboxypeptidase A was routinely assayed with 1 mM hippuryl-L-phenylalanine (Folk and Schirmer, 1963). However, when the degree of association of enzyme and inhibitor was estimated and when the inhibitor was used as an active site titrant for carboxypeptidase A, chloroacetyl-L-tyrosine was used as substrate. Hydrolysis was initiated by the addition of 0.1 ml of 30 mM chloroacetyl-L-tyrosine (pH 7.0) to 3.2 ml of enzyme solution (ca. 1.0 μM) in 1 M NaCl, 50 mM Tris-HCl, pH 7.5. Enzymatic activity was estimated at 25 °C by monitoring the decrease in absorbance at 224 nm as the substrate is hydrolyzed.

Chloroacetyl-L-tyrosine was chosen as substrate in these applications for the following reasons. (1) Substrate concentrations less than K_m (ca. 10 mM; Snoke and Neurath, 1949) are used. Thus substrate would not appreciably perturb the association between enzyme and inhibitor. (2) Concentrations of enzyme and inhibitor greater than the K_I value of 4×10^{-9} M (Ryan et al., 1974) may be employed, allowing nearly complete association of enzyme and inhibitor.

Titration of carboxypeptidase A with the carboxypeptidase inhibitor using hippuryl-L-phenylalanine (1 mM) as substrate is presented in Figure 1. The low enzyme concentrations and possible competition between substrate (K_m 1×10^{-3} M; Pétra, 1970) and inhibitor result in 35% residual activity at equimolar enzyme and inhibitor concentrations. In contrast, the carboxypeptidase inhibitor stoichiometrically inactivates enzyme when chloroacetyl-L-tyrosine is used (Figure 1).

Binding of Arsanilazocarboxypeptidase A. Circular dichroism spectra were recorded at 23 °C on a Cary 60 recording spectropolarimeter equipped with a Model 6001 CD attachment. Spectra were measured on arsanilazocarboxypeptidase A (43 μM) alone and in the presence of 0.75, 1.0, and 1.3 mol ratios of carboxypeptidase inhibitor in 0.5 M NaCl, 50 mM Tris-HCl, pH 7.7, over the wavelength range 400–650 nm. The spectrum of arsanilazocarboxypeptidase A which had been treated with BrAc(Me)Phe was similarly determined.

Gel Filtration. The association of unmodified inhibitor and N^ϵ-[^{14}C]acetyl inhibitor with apocarboxypeptidase A was demonstrated by gel filtration on a 1.6 × 65 cm column of Sephadex G-50 F equilibrated and eluted with 0.1 mM 1,10-phenanthroline in 1 M NaCl, 50 mM Tris-HCl, pH 7.5. Apocarboxypeptidase A (17 mg) and a twofold molar excess of inhibitor were mixed and applied to the column. Fractions (6 ml) were collected and monitored for enzymatic activity toward hippuryl-L-phenylalanine and chloroacetyl-L-tyrosine in the presence of excess $ZnCl_2$ (vide supra). Protein was monitored by the Lowry method (Lowry et al., 1951) when native inhibitor was used, while radioactive inhibitor was detected by counting 200-μl aliquots of each fraction (vide supra).

The association of inhibitor with Ac(Me)Phe·CPA was

[1] Abbreviations used: CPA, carboxypeptidase A; BrAc(Me)Phe, *N*-bromoacetyl-*N*-methyl-L-phenylalanine; Ac(Me)Phe·CPA, carboxypeptidase A that has been treated with *N*-bromoacetyl-*N*-methyl-L-phenylalanine; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; Tos-PheCH$_2$Cl, L-1-tosylamido-2-phenylethyl chloromethyl ketone.

demonstrated by gel filtration. A solution (1.05 ml) containing inhibitor (21 μM) and a 1.4-fold molar excess Ac(Me)Phe·CPA was applied to a 1.4 × 60 cm column of Sephadex G-25 F equilibrated at 4 °C with 1 M NaCl, 50 mM Tris-HCl, pH 7.5. The column was eluted at a rate of 22 ml/h. Fractions (1.1 ml) were monitored for protein by absorbance at 224 nm. Samples containing only enzyme and only inhibitor were chromatographed similarly to determine their characteristic elution patterns.

Competition between Carboxypeptidase A and BrAc-(Me)Phe·CPA for Inhibitor. Competition experiments were conducted by preincubating equimolar mixtures of enzyme and inhibitor (0.28 μM) with increasing amounts of Ac(Me)Phe·CPA in 1 M NaCl, 50 mM Tris-HCl, pH 7.5, for 1 h at 25 °C and then estimating free carboxypeptidase using chloroacetyl-L-tyrosine as substrate. Preliminary experiments indicated that the system attained equilibrium within 1 h. Residual activities of the enzyme in the presence of equimolar concentration of inhibitor (less than 4%, Figure 1) and of Ac(Me)-Phe·CPA (less than 1% of unmodified enzyme) were subtracted from activities observed in competition experiments.

The concentration of free enzyme, $[CPA]_{free}$, in the reaction mixture was determined by assaying with chloroacetyl-L-tyrosine, while the concentrations of other species were calculated from conservation equations as follows.

$$[CPA\text{-}I] = [CPA]_{total} - [CPA]_{free} \quad (1)$$

Since the concentration of free inhibitor is negligible under the experimental conditions, eq 2 may be used to estimate the concentration of the Ac(Me)Phe·CPA–inhibitor complex, where I represents the inhibitor.

$$[Ac(Me)Phe \cdot CPA\text{-}I] = [I]_{total} - [CPA\text{-}I] \quad (2)$$

The difference in free energy of association of the inhibitor with native enzyme relative to [Ac(Me)Phe·CPA] is given by eq 3.

$$\Delta\Delta G = -RT \ln \frac{[CPA\text{-}I][Ac(Me)Phe \cdot CPA]}{[CPA][Ac(Me)Phe \cdot CPA\text{-}I]} \quad (3)$$

Results

Binding of Inhibitor to Arsanilazocarboxypeptidase A. The binding of ligands at the active site of arsanilazocarboxypeptidase A can be detected by CD spectroscopy (Kagan and Vallee, 1969). The minimum in the CD spectrum of this derivative at 515 nm (Figure 2), attributed to a complex between the active site zinc and arsanilazotyrosine 248, is abolished by the binding of competitive inhibitors (Kagan and Vallee, 1969). Modification of enzyme by BrAc(Me)Phe also abolishes the absorbance around 515 nm. This observation precludes the possibility that only binding at a "second site" produced the change in the CD spectrum and demonstrates that binding of ligands in a productive mode disrupts the azotyrosine 248–zinc complex.

The CD spectra of arsanilazocarboxypeptidase A in the presence of increasing amounts of the carboxypeptidase inhibitor are presented in Figure 2. A progressive increase in ellipticity at 515 nm with increasing inhibitor concentration is observed. This spectral change is essentially complete at equimolar concentration of inhibitor and arsanilazoenzyme (Figure 2). The CD spectrum of the arsanilazocarboxypeptidase A–inhibitor complex (Figure 2) is similar to that of enzyme in the presence of substrates and inhibitors, indicating that the inhibitor also disrupts the azotyrosine 248–zinc

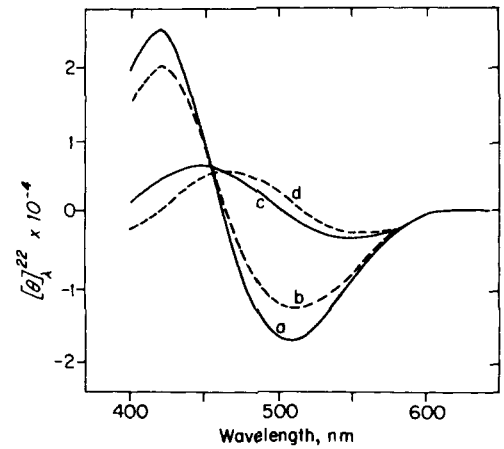

FIGURE 2: Circular dichroism spectra of arsanilazocarboxypeptidase A in the presence of carboxypeptidase inhibitor. Spectra were recorded at 23 °C in 0.5 M NaCl, 50 mM Tris-HCl, pH 7.7, of arsanilazocarboxypeptidase A (43 μM) in the presence of the following concentrations of inhibitor: (curve a) none; (curve b) 32 μM; (curve c) 43 μM; and (curve d) 57 μM.

complex upon binding and probably binds in the active site region.

Binding of Inhibitor to Apocarboxypeptidase A. Gel filtration demonstrates the binding of acetylated carboxypeptidase inhibitor to apoenzyme. These experiments take advantage of the following facts. (1) Carboxypeptidase inhibitor (molecular weight 4000) and apocarboxypeptidase A (molecular weight 34 600) are well resolved on a column of Sephadex G-50. (2) An equimolar mixture of enzyme and inhibitor exhibits approximately 30% the activity of free enzyme when assayed with hippuryl-L-phenylalanine (see Experimental Section; Figure 1). (3) Acetylation of the inhibitor has no effect on the dissociation constant of the complex (Hass et al., 1974, 1976). When a mixture of enzyme and a two-fold molar excess of ^{14}C-acetylated inhibitor is chromatographed on Sephadex G-50 F, two peaks of radioactivity are observed (Figure 3a). The lower molecular weight fraction is free acetylated inhibitor. The higher molecular weight fraction is the apoenzyme–inhibitor complex since this fraction exhibits approximately the same elution volume as apoenzyme yet contains a stoichiometric amount of bound ^{14}C inhibitor. After addition of 0.1 mM $ZnCl_2$, this fraction is active toward hippuryl-L-phenylalanine (about one-third as active as native or reconstituted enzyme) but exhibits no detectable activity against chloroacetyl-L-tyrosine. These properties are consistent with those to be expected for the enzyme–inhibitor complex (see Experimental Section).

A single Lowry-positive peak corresponding to the elution volume of apocarboxypeptidase A is observed when apoenzyme is mixed with a twofold molar excess of unmodified carboxypeptidase inhibitor and chromatographed on a column of Sephadex G-50 F (Figure 3b). The enzymatic activity of this fraction toward hippuryl-L-phenylalanine upon addition of zinc suggests that it is the apoenzyme–inhibitor complex. A peak corresponding to free inhibitor is not seen, presumably because it is present in low amounts.

The reactivation of apoenzyme is nearly instantaneous upon addition of zinc. Since certain peptide substrates prevent the binding of zinc to apocarboxypeptidase A (Coleman and Vallee, 1962), the binding of the carboxypeptidase inhibitor to apoenzyme seems to differ in kind from the binding of peptide substrates.

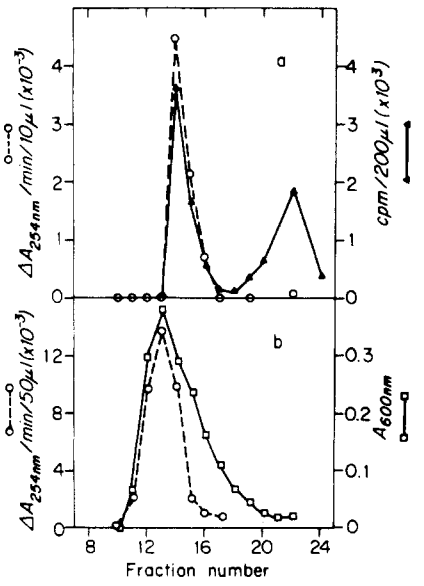

FIGURE 3: Gel filtration of apocarboxypeptidase A and the carboxypeptidase inhibitor in the presence of 0.1 M 1,10-phenanthroline on a column (1.6 × 65 cm) of Sephadex G-50 F. Fractions (6 ml) were collected and assayed with hippuryl-L-phenylalanine (O - - - O) as described in the text. (a) Carboxypeptidase A and twofold excess N^{ϵ}-[^{14}C]acetyl inhibitor. Radioactivity measurements (▲—▲) were made on aliquots (200 μl) of each fraction. (b) Carboxypeptidase A and twofold excess unmodified inhibitor. Protein determinations (□-□) were by the Lowry method (Lowry et al., 1951).

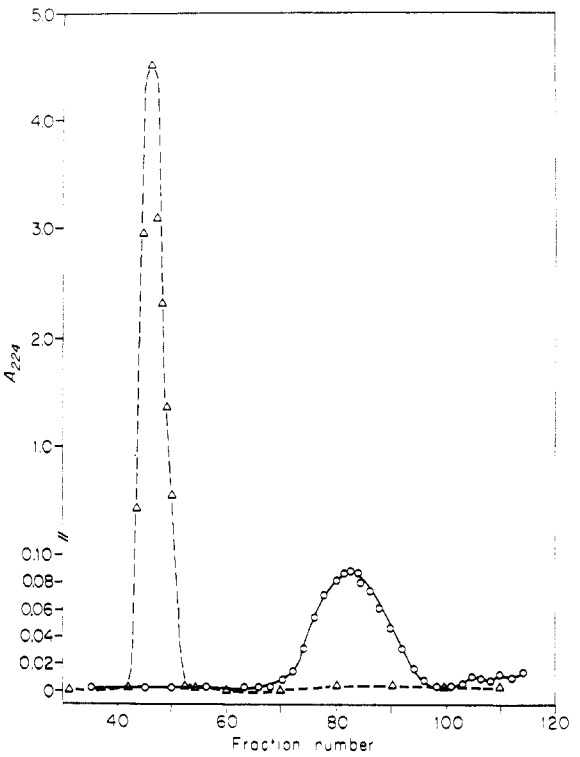

FIGURE 4: The association of the carboxypeptidase inhibitor and Ac(Me)Phe·CPA as demonstrated by gel filtration. Solutions (1.0 ml) of the inhibitor alone (20 μM) (O—O) and inhibitor and a 1.4-fold excess Ac(Me)Phe·carboxypeptidase A (Δ - - - Δ) were chromatographed on a 1.5 × 60 cm column of Sephadex G-25 F. Fractions (1.1 ml) were collected and monitored for protein by absorbance at 224 nm.

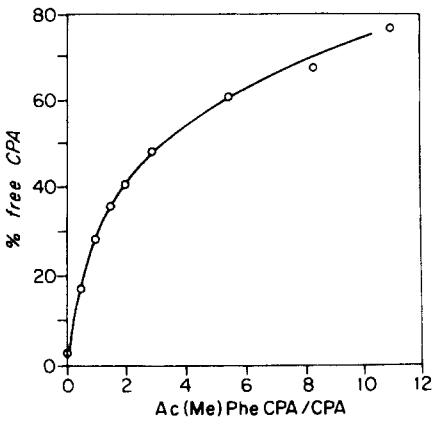

FIGURE 5: Competition between carboxypeptidase A and Ac(Me)Phe·CPA for the inhibitor. Stoichiometric amounts (0.28 μM) of carboxypeptidase A and the inhibitor were equilibrated with varying concentrations of Ac(Me)Phe·CPA at 25 °C in 1 M NaCl, 50 mM Tris-HCl, pH 7.5. Free carboxypeptidase in these mixtures was determined by assaying with chloroacetyl-L-tyrosine.

Binding of Inhibitor to Ac(Me)Phe·CPA. The formation of a complex between Ac(Me)Phe·CPA and the carboxypeptidase inhibitor is demonstrated by gel filtration. When the inhibitor and a 1.4-fold molar excess of modified enzyme are chromatographed on Sephadex G-25 F (Figure 4), no free inhibitor is detected. A quantitative estimate of the strength of binding of inhibitor to Ac(Me)Phe·CPA is provided by competition of this derivative with unmodified enzyme for inhibitor. Stoichiometric amounts of enzyme and inhibitor are preincubated with increasing mole ratios of Ac(Me)Phe·CPA and the equilibrium mixture is then assayed for free enzyme. The results of these experiments are presented in Figure 5. Under these conditions the enzyme-inhibitor complex exhibits 96% inhibition in the absence of Ac(Me)Phe·CPA. Increasing amounts of free enzyme are detected with increasing Ac(Me)Phe·CPA concentrations giving 30% activity at a 1:1 ratio of enzyme to the Ac(Me)Phe derivative. Binding constants calculated from each point according to eq 2 and 3 reveal that the dissociation constant for the enzyme-inhibitor complex is about three times smaller than that of the Ac(Me)-Phe·enzyme-inhibitor complex. This difference in binding constant corresponds to a difference in free energy of association of inhibitor with Ac(Me)Phe·CPA compared with native enzyme of only about 0.4 kcal/mol. Since this represents a small difference compared with the free energy of association of enzyme and inhibitor (−11 kcal/mol) calculated from K_I values, introduction of the Ac(Me)Phe moiety into the active site of the enzyme has little effect on its binding to inhibitor.

Discussion

In contrast to the detailed understanding of the "serine" protease-proteinase inhibitor interactions (Laskowski and Sealock, 1971), relatively little is known of the mechanisms employed by the carboxypeptidase inhibitors. In an earlier report (Hass et al., 1976), the effects of chemical modification of the carboxypeptidase inhibitor from potatoes on the strength of binding to carboxypeptidases A and B were discussed. These studies demonstrate both that the carboxyl-terminal region of the inhibitor interacts with enzyme and that structural integrity of the inhibitor is required for binding to enzyme. In addition, the parallel effects of each chemical modification on binding to carboxypeptidases A and B suggest that these enzymes bind to the same site on the inhibitor.

In this report the binding of carboxypeptidase inhibitor to derivatives of carboxypeptidase A is explored to elucidate the mechanism of inhibition in this system, thus affording a means

of comparison with "serine" protease–proteinase inhibitor interactions.

Based both upon solution chemistry (Vallee and Riordan, 1968) and x-ray diffraction analyses (Lipscomb et al., 1968), catalytic roles in the hydrolysis of peptide substrates by carboxypeptidase A have been ascribed to tyrosine 248, glutamic acid 270, and the zinc atom at the active site. Tyrosine 248 may be specifically modified by treatment with diazotized arsanilic acid forming a catalytically active derivative. The arsanilazotyrosine 248 derivative exhibits a negative band of ellipticity centered approximately at 515 nm which is attributed to a complex between arsanilazotyrosine and the zinc at the active site (Johansen and Vallee, 1971). This band of ellipticity is lost upon disruption of this complex by the presence of inhibitors or substrates, crystallization of the enzyme, or removal of the zinc by treatment with chelating agents (Kagan and Vallee, 1969; Johansen and Vallee, 1973). The binding of carboxypeptidase inhibitor to arsanilazocarboxypeptidase A also produces a disruption of the complex with zinc, as indicated by loss of the ellipticity at 515 nm (Figure 2). These data suggest that the binding of inhibitor occurs in the active site region of the enzyme.

The inhibitor also binds to apocarboxypeptidase as demonstrated by co-chromatography of inhibitor and apoenzyme on a column of Sephadex G-50 F in buffers containing 1,10-phenanthroline (Figures 3a,b). Although the strength of binding of inhibitor to apoenzyme is not known, the interaction does not depend upon the presence of the zinc atom at the active site. The lack of specific interaction of the inhibitor with zinc was not unexpected since excess zinc does not reverse enzyme inhibition (Ryan, 1971) and the inhibitor does not affect the activity of all zinc-containing carboxypeptidases (Ryan et al., 1974). The binding of inhibitor to apoenzyme demonstrates that, as in the case of the "serine" protease inhibitors mentioned previously, binding is not contingent upon enzyme activity. Apocarboxypeptidase could be considered analogous to anhydrotrypsin, anhydrochymotrypsin, or methylchymotrypsin in that these derivatives are catalytically inactive and yet do not have greatly altered binding sites (Ako et al., 1974; Ryan and Feeney, 1975).

BrAc(Me)Phe alkylates the γ carboxylate of glutamic acid 270 at the active site of carboxypeptidase A forming a derivative in which the side chain of the *N*-methylphenylalanine moiety presumably occupies the binding pocket. Since derivatives of the "serine" proteases containing blocked binding sites (e.g., diisopropylchymotrypsin, Tos-PheCH_2Cl-chymotrypsin) interact weakly with proteinase inhibitors (Laskowski and Sealock, 1971; Feinstein and Feeney, 1966), the tight binding between the inhibitor and Ac(Me)Phe·CPA, as demonstrated by competition experiments, was unexpected. The slight increase (0.4 kcal/mol) in free energy of binding of the inhibitor to enzyme attending introduction of the affinity label suggests that interactions of the inhibitor with residues in the enzyme binding pocket do not contribute significantly to the stability of the complex. Thus, the binding of the inhibitor to carboxypeptidases A and B is believed to involve its interaction with structurally similar regions of these enzymes exterior to the binding pocket.

Several modes of binding of the inhibitor to the carboxypeptidases are possible which would disrupt the arsanilazotyrosine–zinc complex without utilizing the enzyme binding pocket. However, two models are consistent with the binding characteristics of small molecules to carboxypeptidase A observed by Hartsuck and Lipscomb (1971). The carboxyl-terminal three residues of the inhibitor are depicted in Figure 6,

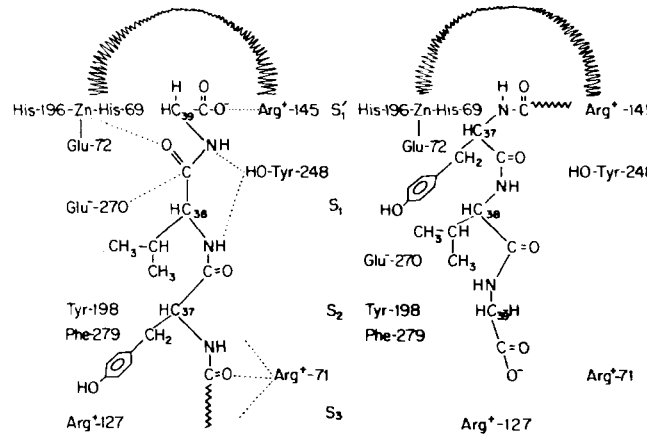

FIGURE 6: Two possible modes of binding of the inhibitor to carboxypeptidase A. The carboxyl-terminal region of the inhibitor (Tyr-Val-Gly-OH) is depicted as interacting with the active site of carboxypeptidase A in (a) the productive substrate binding mode and (b) the nonproductive substrate inhibitor mode (Hartsuck and Lipscomb, 1971).

both to facilitate visualization of the possible interactions and because tyrosine 37 and the α carboxylate of glycine 39 have been shown to interact with enzyme in the complex (Hass et al., 1976).

Figure 6a depicts the enzyme–inhibitor complex, assuming that the carboxypeptidase inhibitor is bound as a peptide substrate. The α carboxylate of glycine 39 of the inhibitor would be in salt linkage to arginine 145 and a hydrophobic bond would occur between tyrosine 37 of the inhibitor and tyrosine 198 and/or phenylalanine 279 of the enzyme. Such a system would disrupt the arsanilazotyrosine–zinc complex, and since the side chain of glycine 39 is a hydrogen atom, the occupation of the enzyme binding pocket by the phenylalanine ring of the affinity label, BrAc(Me)Phe, should not greatly affect binding strength. However, two possible difficulties with this model must be noted. First, the α carboxylate of glycine 39 would be in close proximity to the α carboxylate of the affinity label in Ac(Me)Phe·CPA producing charge repulsion which might be expected to significantly weaken enzyme–inhibitor interaction. This would contradict the similarity in binding strengths of carboxypeptidase inhibitor to unmodified enzyme and the carboxypeptidase inhibitor Ac(Me)Phe derivative of the enzyme. In addition, it might be expected that the binding of the carboxypeptidase inhibitor in the "correct" substrate binding mode would lead to hydrolysis of the Val_{38}–Gly_{39} peptide bond. This hydrolysis is not believed to occur since untreated inhibitor and inhibitor which has been incubated with carboxypeptidase A have identical amino acid compositions.[2]

The second and more attractive model for enzyme–inhibitor interaction is presented in Figure 6b. The α carboxylate of the inhibitor would be in salt linkage with the guanido group of arginine 127 of the enzyme, and valine 38 and tyrosine 37 would form hydrophobic bonds with phenylalanine 279 and tyrosine 198. The model would predict displacement of tyrosine 248 upon binding inhibitor and is consistent with the observed binding strength between carboxypeptidase inhibitor and Ac(Me)Phe·CPA. In addition, the structural similarity between carboxypeptidases A and B in the proposed contact region is consistent with the similar binding strengths of carboxypeptidase inhibitor to these enzymes.

[2] G. M. Hass, unpublished observations.

The model presented in Figure 6b is highly speculative, particularly in view of recent reports (Johansen and Vallee, 1971, 1973; Spilburg et al., 1974) which question the validity of the active site structure and mechanism of action of carboxypeptidase A proposed by Lipscomb et al. (1968). Clearly, detailed understanding of the mode of binding of this inhibitor to target enzymes must await further experimentation. However, the present studies do allow a comparison with certain "serine" protease–proteinase inhibitor interactions. In both types of systems, enzymatic activity is not required for binding of inhibitor even though inhibitor binding involved the enzyme active site region (Ako et al., 1974; Laskowski and Sealock, 1971). The principal difference between these systems is that a significant contribution to the free energy of binding between "serine" proteases and their inhibitors is due to a specific interaction between the side chain of the carboxyl donor of the reactive site of the inhibitor with the enzyme binding pocket (Rigbi, 1971; Ruhlmann et al., 1973; Sweet et al., 1974), while interaction of carboxypeptidase inhibitor with target enzymes apparently does not involve similar binding site contacts.

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