Carboxypeptidase Inhibitor from Potatoes. The Effects of Chemical Modifications on Inhibitory Activity†

G. Michael Hass,*,‡ Harry Ako,§ Donald T. Grahn, and Hans Neurath

ABSTRACT: The carboxypeptidase inhibitor from Russet Burbank potatoes was subjected to a variety of chemical modifications and their effects on inhibitory activity toward carboxypeptidases A and B were determined. The importance of the α carboxylate of glycine-39 to the enzyme–inhibitor interaction was demonstrated by the observation that a derivative in which all four carboxyls were modified was inactive whereas a derivative in which only the β carboxylates of aspartic acid residues 5, 16, and 17 were masked retained full inhibitory activity. In addition to these three aspartic acid residues, lysine residues 10 and 13, histidine residues 3 and 15, and arginine-32 were modified and residues 1–5 removed with little effect on inhibitory activity. Tryptophan residues 22 and 28 did not react with 2-hydroxy-5-nitrobenzyl bromide or *o*-nitrophenylsulfenyl chloride, and thus are presumed to be buried in the interior of the inhibitor molecule. Although tyrosine-37 was acetylated without affecting binding characteristics, both carboxypeptidases A and B protected against deacetylation by hydroxylamine. These studies indicate that the carboxyl terminal region of the inhibitor is in contact with enzyme in the complex. The parallel effects of modifications on inhibitory activity toward carboxypeptidases A and B support previous evidence that both enzymes utilize the same binding site on the inhibitor [C. A. Ryan (1971), *Biochem. Biophys. Res. Commun. 44*, 1265].

The chemical and physical properties (Ryan et al., 1974) and amino acid sequence (Hass et al., 1975) of a carboxypeptidase inhibitor from Russet Burbank potatoes were recently described. This inhibitor, effective toward both carboxypeptidases A and B ($K_I \sim 1 \times 10^{-9}$ *M*), is a mixture of two closely related polypeptides of 38 and 39 amino acid residues (Figure 1).

This report presents the effects of a variety of chemical modifications of the inhibitor on the strength of binding to carboxypeptidases A and B. These studies identify a region of the inhibitor in contact with enzyme in the complex and suggest that both carboxypeptidases A and B bind to this site on the inhibitor.

Materials and Methods

Materials. The carboxypeptidase inhibitor was prepared from Russet Burbank potatoes as described previously (Ryan et al., 1974) except that SE-cellulose (Gallard Schlesinger) and Sephadex G-50 F were substituted for phosphocellulose and Sephadex G-75, respectively. Bovine carboxypeptidases A and B were prepared from activated pancreatic juice (Reeck et al., 1971). Yeast protease C (carboxypeptidase Y) was the generous gift of Dr. Paul Levy. Trypsin treated with L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone was purchased from Worthington Biochemical Corp.; hippuryl-L-phenylalanine and hippuryl-L-arginine·$2H_2O$ were purchased from Fox Chemical Company; Sephadex G-25 SF, Sephadex G-50 F, and SE-Sephadex C-25 from Pharmacia Fine Chemicals; 2,4,6-trinitrobenzenesulfonic acid and *o*-nitrophenylsulfenyl chloride from Pierce; phenylglyoxal monohydrate, 1-acetylimidazole, 2-hydroxy-5-nitrobenzyl bromide, and L-leucine methyl ester hydrochloride were from Aldrich; [1-^{14}C]acetylimidazole with a specific radioactivity of 22.3 Ci/mol was from New England Nuclear.

Analytical Procedures. The concentrations of stock solutions of the carboxypeptidase inhibitor and its derivatives were determined by amino acid analysis (Spackman et al., 1958) after hydrolysis in 6 *N* HCl for 18–24 hr at 110° (Moore and Stein, 1963).

Concentrations of carboxypeptidase A and B solutions were calculated assuming A_{278}(0.1%) of 1.88 (Bargetzi et al., 1963) and 2.1 (Cox et al., 1962), respectively.

Radioactivity measurements were performed with a Packard TriCarb liquid scintillation spectrometer, Model 3003. The scintillation fluid was toluene–Triton X-100 (2:1, v/v containing 2.67 g/l. of 2,5-diphenyloxazole and 0.067 g/l. of 1,4-bis[2-(5-phenyloxazolyl)]benzene). Radioactive toluene was introduced as an internal standard to correct for quenching.

Absorbance measurements and enzyme assays were performed on a Gilford Model 2000 spectrophotometer.

Enzyme Assays. Carboxypeptidase A was routinely assayed spectrophotometrically with hippuryl-L-phenylalanine as substrate (Folk and Schirmer, 1964). The change in absorbance at 254 nm was monitored at 25° in 3 ml of solutions containing substrate in 0.5 *M* NaCl–0.05 *M* Tris-HCl (pH 7.5).

When titrating inhibitor with carboxypeptidase A, chloroacetyl-L-tyrosine (1 m*M*) was employed as substrate in 1.0 *M* NaCl–0.05 *M* Tris-HCl (pH 7.5) by monitoring a decrease in absorbance at 224 nm as the amide bond was hydrolyzed.

Carboxypeptidase B activity was monitored by the increase in absorbance at 254 nm as hippuryl-L-arginine was hydrolyzed. A suitable aliquot of enzyme was added to 3 ml

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‡ Investigator of the Howard Hughes Medical Institute. Present address: Department of Bacteriology and Biochemistry, University of Idaho, Moscow, Idaho 83843.

§ Present address: Department of Agricultural Biochemistry, University of Hawaii, Honolulu, Hawaii 96822.

of substrate in 0.5 *M* NaCl–0.05 *M* Tris-HCl (pH 7.5 at 25°) (Wintersberger et al., 1962).

K_I Values. K_I values for the derivatives of the inhibitor generated by cleavage of the aspartyl-proline bond, by acetylation of the ϵ-amino groups, and by treatment with phenylglyoxal were determined by the method of Lineweaver and Burk (1934). Substrate concentrations were varied from 0.125 to 1.0 m*M* in assay buffer and initial velocities were determined in the presence and absence of inhibitor (20–50 n*M*). For these analyses molar ratios of inhibitor/enzyme were 5 or greater for carboxypeptidase A, and 10 or greater for carboxypeptidase B.

Estimations of K_I values for the native inhibitor, the *O*-acetyltyrosine derivative, and the carboxamidomethylhistidine derivative were performed as described by Henderson (1972, 1973). Inhibitor concentrations were varied from 10 to 200 n*M* at 1 m*M* substrate concentration. For analysis, $I_i/(1 - v_i/v_o)$ was plotted against v_o/v_i where I_i is the concentration of inhibitor, v_o is the observed velocity in the absence of inhibitor, and v_i the velocity in the presence of inhibitor. K_m values of 1.67 m*M* (carboxypeptidase A) and 0.31 m*M* (carboxypeptidase B) for hippuryl-L-phenylalanine and hippuryl-L-arginine, respectively, as determined by Lineweaver–Burk analysis (vide supra), were employed in calculating K_I values from K_I app.

Preparation and Characterization of Derivatives. Tetraleucyl Inhibitor. The inhibitor (30 mg) was dissolved in 6 ml of 6 *M* guanidine hydrochloride and 1 *M* leucine methyl ester and the solution adjusted to pH 4.75. Solid 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride was added to give a final concentration of 0.1 *M* and the pH maintained at 4.75 for 2 hr (Hoare and Koshland, 1967). The modified inhibitor was desalted by gel filtration on a 1.5 × 80 cm column of Sephadex G-25 F equilibrated and eluted with 5% formic acid. Fractions containing polypeptide were pooled and lyophilized. The methyl ester groups were removed by incubating the modified inhibitor at pH 10.0 for 24 hr at 25°. The tetraleucyl derivative was desalted as described above and lyophilized.

Correlation of leucine residues released by carboxypeptidase Y digestion with regain of inhibitory activity was determined as follows. Aliquots (200 μl) of tetraleucyl inhibitor (0.36 m*M*) in 0.05 *M* Mes[1] buffer (pH 6.0) were digested for various times at 25° with carboxypeptidase Y (1:20 molar ratio). Digestions were terminated by the addition of 1 ml of 0.2 *M* sodium citrate (pH 2.2) and 1-ml aliquots subjected to amino acid analysis. Blanks containing only enzyme and only inhibitor were also prepared and analyzed.

The remainder of each solution was tested for inhibitory activity toward carboxypeptidase A by incubating inhibitor derivatives and an equimolar concentration of enzyme (0.39 μ*M*) in 3.12 ml of 1 *M* NaCl–0.05 *M* Tris-HCl (pH 7.5). Assays were performed at 25° by following a decrease in absorbance at 224 nm after the addition of 100 μl of 0.03 *M* chloroacetyl-L-tyrosine. Inhibitory activity toward carboxypeptidase B was determined by assaying 20-μl aliquots of inhibitor solutions (60 μ*M*) with 1 m*M* hippuryl-L-arginine (vide supra).

For peptide analysis 20-mg samples of tetraleucyl inhibitor were treated with carboxypeptidase Y (2 hr followed by 1 hr after the addition of fresh enzyme). These samples were reduced and *S*-carboxymethylated (Hass et al., 1975), desalted by gel filtration (vide supra), and lyophilized. These preparations (10 mg each) were dissolved in 2.0 ml of 0.1 *M* Mops (pH 7.5) at 0° and treated with 30 μl of acetic anhydride for 1 hr at pH 7.5. After incubation at pH 10.0 for 1 hr to destroy excess acetic anhydride, the pH was lowered to pH 7.5 and 0.1 mg of TosPheCH$_2$Cl-treated trypsin added to initiate cleavage at arginine-32. Digestion at 37° was terminated after 15 min by the addition of 10 μl of 1 *M* diisopropyl phosphofluoridate (in 2-propanol). Peptides T-2 (residues 33–39) derived from untreated and carboxypeptidase Y-treated tetraleucyl inhibitor were purified by gel filtration on Sephadex G-25 SF in 0.01 *M* ammonium bicarbonate (Hass et al., 1975) and characterized by amino acid analysis.

O-Acetyltyrosine Inhibitor. The inhibitor (10 mg) was treated with a 60-fold molar excess of acetylimidazole (5 μCi of [1-^{14}C]acetylimidazole, specific activity 22.28 Ci/mol, diluted with 8 mg of unlabeled reagent) in 1.2 ml of 0.02 *M* sodium Veronal (pH 7.5) for 1 hr at room temperature (Riordan and Vallee, 1967). The acetylated peptide was desalted on a 1.5 × 75 cm column of Sephadex G-25 SF equilibrated with 0.01 *M* ammonium bicarbonate. Elution was monitored by absorbance at 280 nm and by radioactivity determinations on 200-μl aliquots. Fractions containing peptide were pooled and lyophilized.

The extent of acetylation of tyrosine and the rates of deacetylation in the presence and absence of an equimolar concentration of carboxypeptidases A and B were determined by monitoring the change in absorbance at 278 nm upon treatment of the modified inhibitor with 50 m*M* hydroxylamine in 1 *M* NaCl–0.01 *M* Mops (pH 7.5 at 25°). The extent of modification was determined assuming $\Delta\epsilon_{278} = 1160$ for the hydrolysis of *O*-acetyltyrosine (Riordan and Vallee, 1967).

The extent of acetylation of lysine under these conditions was estimated by treating the acetylated inhibitor with hydroxylamine (vide supra) and separating the inhibitor from acetohydroxamate by gel filtration on a 1.5 × 75 cm column of Sephadex G-25 SF equilibrated with 0.01 *M* ammonium bicarbonate. Radioactivity remaining in the polypeptide fraction after hydroxylamine treatment was assumed to be associated with acetylated lysine residues.

Carboxamidomethyl Inhibitor. The inhibitor (16 mg) was incubated in 4.0 ml of 0.1 *M* iodoacetamide–0.1 *M* sodium phosphate (pH 7.2) at room temperature in the dark. At various times 0.5-ml aliquots were desalted on a 0.9 × 40 cm column of Sephadex G-25 F with 0.01 *M* ammonium bicarbonate (pH 7.2) and lyophilized. The amount of unmodified histidine in each sample was determined by amino acid analysis based upon one residue of arginine per molecule.

S-Cm Inhibitor. The *S*-carboxymethyl derivative of the inhibitor was prepared by the method of Crestfield et al. (1963) as described previously (Hass et al., 1975).

Phenylglyoxylarginine Inhibitor. The inhibitor (2.5 mg) was dissolved in 0.25 ml of 0.2 *M* *N*-ethylmorpholineacetic acid (pH 8.0). Phenylglyoxal (0.25 ml of a 1.5% solution) was added to initiate the reaction at 25° (Takahashi, 1968). Aliquots (50 μl) were withdrawn at various times and hydrolyzed with 6 *N* HCl, and the hydrolysates were subjected to amino acid analysis.

In order to determine K_I values of this derivative, a 6-mg sample of inhibitor was treated with phenylglyoxal for 1 hr (vida supra). The modified inhibitor was separated from excess reagents by gel filtration on a 1.5 × 75 cm column of

[1] Abbreviations used are: Cm, carboxymethyl; Mes, 4-morpholinoethanesulfonic acid; Mops, 4-morpholinopropanesulfonic acid.

Sephadex G-25 F in 0.1 *M* propionic acid. Elution was monitored by absorbance at 280 and 224 nm. Fractions containing peptide were pooled and lyophilized. A stock solution of this derivative was prepared in 0.01 *M* acetic acid.

Fragment AP-2 (Residues 6–39). The inhibitor (20 mg) was dissolved in 1.5 ml of 7 *M* guanidine hydrochloride and 10% acetic acid and the pH of the solution adjusted to 2.5 with pyridine. The solution was incubated at 40° for 4 days to effect cleavage of the Asp_5–Pro_6 bond (Fraser et al., 1972). Peptides AP-1 and AP-2 were separated from reagents by gel filtration on a 1.5 × 75 cm column of Sephadex G-25 F equilibrated and eluted with 0.1 *M* propionic acid (Hass et al., 1975). Elution of peptides was monitored by absorbance at 280 and 224 nm and peptide fractions were pooled and lyophilized. The peptide mixture was dissolved in 4 ml of 0.05 *M* pyridine–acetic acid (pH 2.4) and chromatographed on a 0.9 × 15 cm column of SE-Sephadex C-25 at 50°. Peptides were eluted by a linear gradient comprised of 200 ml each of 0.05 *M* pyridine–acetic acid (pH 2.4) and 2.0 *M* pyridine–acetic acid (pH 5.0). The flow rate was 20 ml/hr. Peptide elution was monitored by automatic base hydrolysis and ninhydrin analysis on 9% of the eluate (Hill and Delaney, 1967).

Three peaks were observed by ninhydrin analysis and the corresponding fractions were pooled and lyophilized. Two of these, SE-2 and SE-3, were identified as peptides AP-1 and AP-2, respectively, by amino acid analysis after acid hydrolysis, while pool SE-1 appeared to be a small amount of intact inhibitor. In addition, peptide AP-2 was subjected to automatic Edman degradation in the Beckman sequencer (Edman and Begg, 1967; Hermodson et al., 1972) and tested for inhibitory activity.

N^{ϵ}-Acetyllysine Inhibitor. The inhibitor (10 mg) was dispersed in 0.4 ml of 50% saturated sodium acetate at 0°. Acetic anhydride (12 μl) was added in 2-μl portions over a 1-hr period (Riordan and Vallee, 1967). After an additional hour, 1 ml of cold water was added which solubilized the modified inhibitor. The derivative was desalted on a 1.5 × 75 cm column of Sephadex G-25 F in 0.1 *M* propionic acid. Appropriate fractions were lyophilized, and again acetylated and desalted as described above. Free amino groups were determined by titration with 2,4,6-trinitrobenzenesulfonate (Fields, 1972). Protein concentration was determined by amino acid analysis and free amino groups estimated assuming a molar absorbance of 19200 M^{-1} cm^{-1} for the trinitrophenyl-ε-amino group.

Tryptophan Modifications. The inhibitor (10 mg) was dissolved in 1 ml of 0.2 *M* sodium acetate at 0°; 50 μl of a 0.2 *M* solution of 2-hydroxy-5-nitrobenzyl bromide in acetone was added in 10-μl aliquots over a 1-hr period. The reaction mixture was diluted with 1 ml of H_2O and centrifuged, and the supernatant applied to a 1.5 × 75 cm column of Sephadex G-25 F in H_2O. Fractions containing peptide were pooled and lyophilized. The extent of modification was estimated from absorbance at 410 nm in 3.0 *N* NaOH assuming ε 18450 M^{-1} cm^{-1} for the phenolate form of the monosubstituted tryptophan derivative (Horton and Koshland, 1965).

Portions of native inhibitor (5 mg) and of the *S*-carboxymethyl derivative were dissolved in 1 ml of 25% acetic acid and treated with a 20-fold excess of *o*-nitrophenylsulfenyl chloride [2 ml of a 2-mg/ml solution in glacial acetic acid (Scoffone et al., 1968)]. The solutions were desalted on a 1.5 × 80 cm column of Sephadex G-25 F in 5% formic acid and fractions containing protein were pooled and lyophilized. The extent of modification was estimated from the absorbance at 265 nm assuming ε 4000 for nitrophenylsulfenyl derivative of tryptophan (Scoffone et al., 1968).

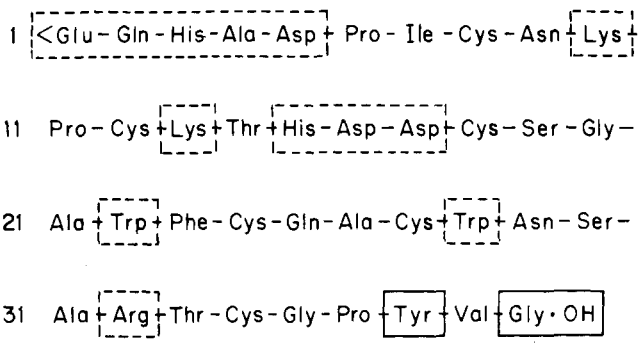

FIGURE 1: Amino acid sequence of carboxypeptidase inhibitor (Hass et al., 1975) and summary of chemical modification studies. Inhibitor preparations consist of an approximately equal mixture of peptides having the amino terminal sequences of <Glu-His-Ala-Asp and <Glu-Gln-His-Ala-Asp. Residues enclosed in dashed lines are believed not to come in contact with enzyme, those enclosed in solid lines are thought to be part of the contact region of the inhibitor.

Table I: Inhibition of Bovine Carboxypeptidases A and B by Derivatives of Carboxypeptidase Inhibitor.

	Carboxypeptidase A		Carboxypeptidase B	
Derivative[a]	K_I (*M*)	Method[b]	K_I (*M*)	Method[b]
Native I	5.2×10^{-9}	H	1×10^{-8}	LB
	5.0×10^{-9} [c]	LB	5×10^{-8} [c]	LB
$(Leu)_4$-I[d]	$>4.0 \times 10^{-6}$	T	$>1 \times 10^{-6}$	T
$(Leu)_3$-I[d]	$<4.0 \times 10^{-8}$	T	$<3 \times 10^{-8}$	T
O-Acetyl-tyrosyl-I	1.4×10^{-9}	H	3×10^{-8}	LB
Carboxamido-methyl-I	2.0×10^{-8}	H	7×10^{-8}	LB
S-Cm-I	$>5.0 \times 10^{-5}$	LB	$>5 \times 10^{-5}$	LB
Phenylglyoxyl-arginine-I	5.0×10^{-9}	LB	2×10^{-8}	LB
AP-2	4.5×10^{-9}	LB	1×10^{-8}	LB
N^{ϵ}-Acetyl-lysine-I	4.4×10^{-9}	LB	2×10^{-8}	LB

[a] Derivatives were prepared and characterized as described in the text. I denotes carboxypeptidase inhibitor. [b] K_I values were estimated by titration (T), Lineweaver–Burk analysis (LB), or by Henderson's treatment (Henderson, 1972, 1973) (H) as described in the text. [c] Data taken from Ryan et al. (1974) for the inhibition of bovine carboxypeptidase A and porcine carboxypeptidase B. [d] $(Leu)_4$-I refers to tetraleucyl inhibitor and $(Leu)_3$-I to tetraleucyl inhibitor which had been treated with carboxypeptidase Y.

Results

Native Inhibitor. The carboxypeptidase inhibitor from potatoes (Figure 1) is an approximately equal mixture of molecules containing 38 and 39 amino acid residues (Hass et al., 1975). K_I values of 5×10^{-9} and 5×10^{-8} *M* were determined previously (Ryan et al., 1974) for the inhibition of bovine carboxypeptidase A and porcine carboxypeptidase B, respectively, employing the method of Lineweaver and Burk (1934). The method of Henderson (1972, 1973) provided another estimate of K_I for the inhibitor–bovine carboxypeptidase A complex (Table I).

The slope of the line relating $I_t/(1 - v_i/v_o)$ and v_o/v_i at different inhibitor concentrations is equal to K_I app (i.e., $K_I(K_m + S/K_m)$ as described in the Experimental Section.

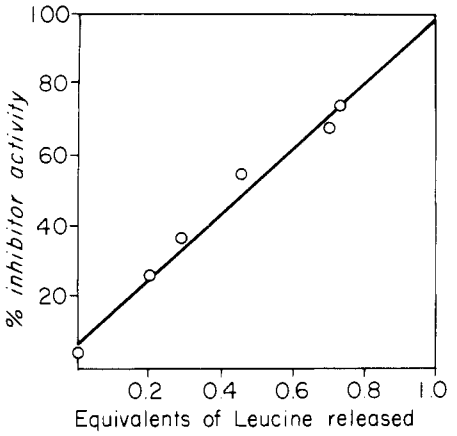

FIGURE 2: Regain of inhibitory activity upon release of leucine from tetraleucyl inhibitor catalyzed by carboxypeptidase Y. Active inhibitor was titrated with carboxypeptidase A as described in the text. Samples were analyzed at 0, 0.33, 1, 5, 30, and 180 min after the addition of carboxypeptidase Y, respectively.

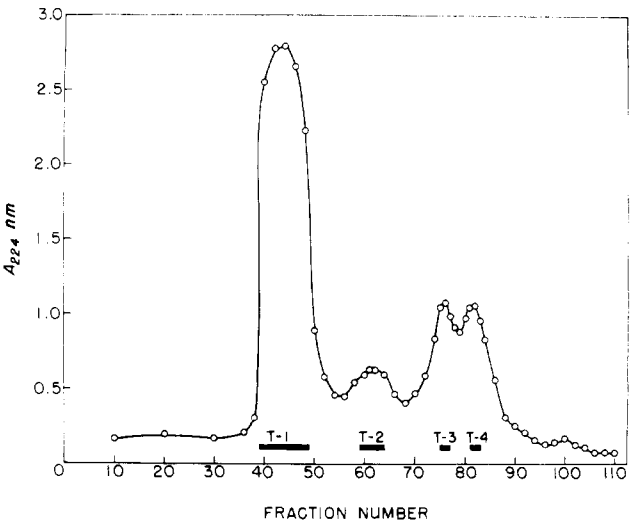

FIGURE 3: Gel filtration on a 1.5 × 80 cm column of Sephadex G-25 SF in 0.01 *M* ammonium bicarbonate of the peptides derived from tetraleucyl inhibitor by cleavage at arginine with trypsin. The sample was reduced and carboxymethylated and then acetylated prior to digestion with trypsin. Elution of peptides was monitored by absorbance at 224 nm (O). A similar pattern was obtained with carboxypeptidase Y-treated tetraleucyl inhibitor as described above.

The K_I value of 5.2×10^{-9} *M* estimated from these data is in excellent agreement with that determined by Lineweaver-Burk analysis (Ryan et al., 1974).

The K_I value of 1×10^{-8} *M* for the inhibition of bovine carboxypeptidase B is somewhat lower than that reported for the inhibition of porcine carboxypeptidase B (Table I). The difference in the strength of binding of the inhibitor to bovine carboxypeptidases A and B is thus only 1.4 kcal/mol.

Tetraleucyl Inhibitor. Preparations usually contained from 3.7 to 4.2 residues of leucine incorporated per molecule of inhibitor, suggesting that the four free carboxylates (i.e., the β carboxylates of aspartic acid residues 6, 15, and 17 and the α carboxylate of glycine-39) were modified. This derivative was incubated at pH 10.0 to remove the methyl ester groups prior to further characterization.

The tetraleucyl derivative had little, if any, inhibitory activity toward carboxypeptidase A (Figure 2). At equal concentrations of enzyme and inhibitor (0.4 μM) approximately 95% of the maximal carboxypeptidase A activity was observed. This procedure would not discriminate between the possibilities that residual inhibitory activity was due to a slight contamination of tetraleucyl inhibitor with unmodified inhibitor or that the derivative bound enzyme weakly. If it is assumed that the observed inhibition resulted from a residual activity of this derivative rather than contamination, K_I values can be estimated. These are given in Table I and are based on the residual 5% carboxypeptidase A activity (Figure 2) and 8% carboxypeptidase B activity observed when assays were performed in the presence of 0.4 μM tetraleucyl inhibitor. Thus, modification of the carboxylates by coupling to leucine increased the K_I by at least three orders of magnitude toward carboxypeptidase A.

Incubation of tetraleucyl inhibitor with carboxypeptidase Y, a "serine" carboxypeptidase not affected by the native inhibitor (Ryan et al., 1974), produced a maximum release of nearly one leucine residue per molecule (Figure 2). No further degradation of this derivative by carboxypeptidase Y was observed, since only the leucine value was significantly altered during enzymatic digestion. The release of leucine paralleled regeneration of active inhibitor (Figure 2). In these studies, chloroacetyl-L-tyrosine was employed as substrate for carboxypeptidase A because of the following. (1) It is hydrolyzed slowly by the enzyme allowing the relatively high concentrations of enzyme and inhibitor. These high concentrations diminish the dissociation of the enzyme-inhibitor complex (Ako, H., Hass, G. M., Grahn, D. T., and Neurath, H., in preparation). (2) It may be utilized at concentrations well below its K_m of approximately 10 m*M* (Putnam and Neurath, 1946) obviating the need to correct for competition between substrate and inhibitor in evaluating titration data.

The K_I value for the inhibition of carboxypeptidase B by the tetraleucyl inhibitor previously treated with carboxypeptidase Y (Table I) was calculated from the observed 94% inhibition of activity at a derivative concentration of 0.4 μM. The stoichiometric release of leucine with concomitant regain of inhibitory activity toward carboxypeptidase A (Figure 2) would require a K_I value of less than 4×10^{-8} *M* for the enzyme-treated derivative, since inhibitor concentrations of 3.9×10^{-7} *M* were employed in these assays (see Materials and Methods). A K_I value higher than 4×10^{-8} *M* would produce less than stoichiometric regain of inhibitory activity under these conditions.

Based upon the known substrate specificities of the carboxypeptidases (Pétra, 1970; Kuhn et al., 1974), carboxypeptidase Y would be expected to cleave the peptide bond between the α carboxylate of glycine-39 and the amino group of leucine in preference to the amide bonds between leucine and the β carboxylates of aspartic acid residues 5, 15, and 16. The critical site of modification was identified as the α carboxylate of glycine-39 by comparing the amino acid composition of peptide T-2 (residues 33–39) derived from tetraleucyl inhibitor with that from the carboxypeptidase Y-treated derivative (Table II). The preparation of tetraleucyl inhibitor used for peptide analysis was less highly substituted than typical batches, containing an average of 3.6 residues leucine per molecule. After treatment with carboxypeptidase Y this value decreased to 2.7 residues of leucine per molecule.

Samples of tetraleucyl inhibitor and its carboxypeptidase Y-treated derivative were reduced, carboxymethylated, and then acetylated in preparation for tryptic digestion. The

Table II: Amino Acid Compositions (Residues per Molecule) of Tryptic Peptide T-2 from *S*-Cm Inhibitor, Tetraleucyl Inhibitor, and Tetraleucyl Treated with Carboxypeptidase Y

Amino Acid[a]	*S*-Cm Inhibitor[b]	Tetraleucyl Inhibitor	
		Untreated	Treated[c]
Cm-cysteine	1.0	1.2	0.81
Threonine	1.0	0.90	1.10
Proline	1.0	0.96	1.00
Glycine	2.0	2.0	2.00
Valine	1.0	0.63	0.89
Leucine	0.0	0.90	0.15
Tyrosine	0.9	0.80	0.97

[a] All other amino acids are present at less than 0.2 residue per molecule. [b] Taken from Hass et al. (1975). [c] Incubated with carboxypeptidase Y as described in text.

peptides generated from these derivatives by trypsin-catalyzed cleavage at arginine-32 were separated by gel filtration on Sephadex G-25 SF (Figure 3) and characterized by amino acid analysis. Fraction T-1 contained the amino terminal fragment (residues 1-32) and some intact inhibitor. Fractions T-3 and T-4 contained only trace amounts of peptide material. The amino acid compositions of peptides T-2 (derived from the carboxyl-terminal regions of the inhibitor derivatives) are shown in Table II. The comparison of leucine present in peptide T-2 from tetraleucyl inhibitor (0.9 residue per molecule) with that from the carboxypeptidase Y-digested derivative (0.15 residue per molecule) demonstrated that leucine was released by carboxypeptidase Y from the carboxyl-terminal residue of the polypeptide chain.

Since modification of the α carboxylate of the inhibitor diminishes or eliminates inhibitory activity, peptide T-2 was tested for its effect on carboxypeptidase A. A K_I value for this peptide higher than 4×10^{-4} M was estimated from Lineweaver-Burk plots. These data support the conclusion (vide infra) that the enzyme-inhibitor interaction is more complex than the binding of a specific sequence of amino acids to the enzyme active site.

O-Acetyltyrosine Inhibitor. Tyrosine-37 lies near the carboxyl terminus of the polypeptide chain. Acetylation of the inhibitor by treatment with a 40-fold molar excess of acetylimidazole modified an average of 1.05 tyrosine residues per molecule as judged by the spectral change attending deacetylation by hydroxylamine (Simpson et al., 1963). Hydroxylamine treatment of [1-^{14}C]acetylimidazole modified inhibitor released only 57% of the total radioactivity incorporated suggesting that lysine (approximately 0.8 residue per molecule) had been acetylated in addition to the single residue of tyrosine. K_I values (Table I) for the O-acetylated derivative did not differ greatly from those of the native inhibitor, indicating that the phenolic hydroxyl group does not contribute to the free energy of binding.

However, the location of tyrosine-37 in or near the contact region was suggested by the protection of the *O*-acetyl group by the carboxypeptidases. Whereas deacetylation of tyrosine-37 in 0.05 M hydroxylamine occurred according to first-order kinetics with a half-time of 50 min, inclusion of either carboxypeptidase A or B totally protected against deacetylation under these experimental conditions.

Carboxamidomethyl Inhibitor. The reaction of proteins with α-halo acids and related compounds near pH 7.0 occurs predominantly with histidine residues, provided the protein does not contain cysteine (Gurd, 1967). The time course of the modification of the carboxypeptidase inhibitor, containing two histidine residues per molecule (positions 3 and 15), is presented in Figure 4. Although neither histidine residue appeared particularly reactive, the data suggest that one reacted preferentially. After 56 days of incubation an average of only about 0.25 histidine residue per molecule remained unmodified whereas the remainder was found as the 1,3-dicarboxymethyl derivative after acid hydrolysis. This derivative exhibited a slightly elevated K_I value toward both carboxypeptidases (Table I), suggesting that one or both histidines are in the contact region. These elevated K_I values did not reflect partial denaturation of the inhibitor during prolonged incubation (56 days), since preparations of carboxamidomethyl inhibitor were 95% active when titrated with carboxypeptidase A employing chloroacetyl-L-tyrosine as substrate.

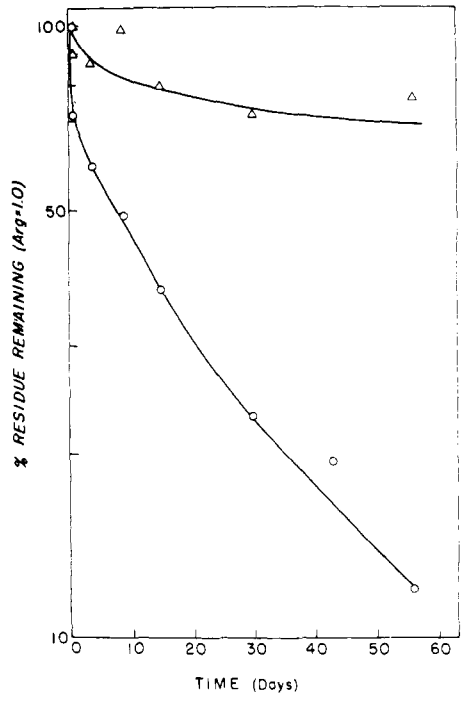

FIGURE 4: Rate of modification of inhibitor (1 mM) upon incubation with 0.1 M iodoacetamide at pH 7.2. The extent of alkylation of histidine (○) and lysine (△) was monitored by amino acid analysis of appropriate aliquots.

S-Cm Inhibitor. The requirement of three-dimensional integrity of inhibitor for inhibitory activity was demonstrated by testing the reduced and *S*-carboxymethylated derivative. At a concentration of 4×10^{-6} M, no inhibition of carboxypeptidase A or B activity was observed. At this concentration of native inhibitor, 100% inhibition was observed. From these observations K_I values higher than 5.0×10^{-5} M were estimated for the inhibition of the carboxypeptidases by *S*-Cm inhibitor (Table I).

Phenylglyoxylarginine Inhibitor. Takahashi (1968) has shown that under the experimental conditions of these studies, phenylglyoxal reacts with the guanido group of arginine to form a derivative containing two residues of phenylglyoxal. Possible side reactions would be the deamination of α-amino groups and, upon extended incubation, the modification of the ϵ-amino group of lysine. Modification of the single arginine residue (position 32) of the carboxypeptidase inhibitor was essentially complete after a 20-min incubation at a reagent concentration of 0.75%. Inhibition studies

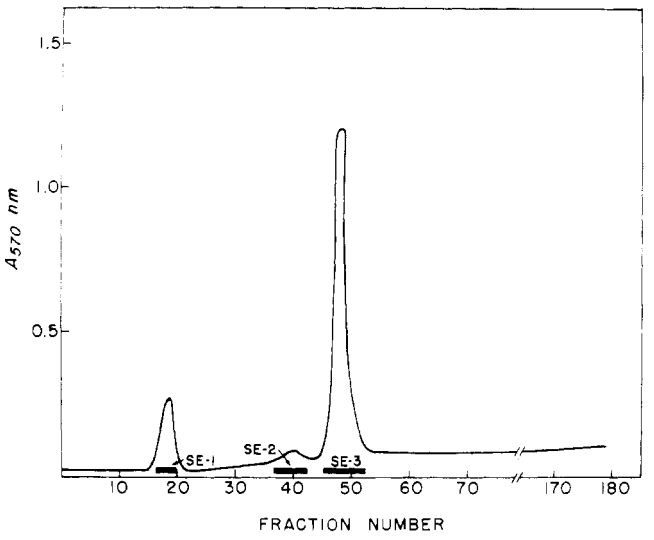

FIGURE 5: Chromatography on a 0.9 × 15 cm column of SE-Sephadex C-25 of peptides generated by cleavage of the Asp_5–Pro_6 bond of inhibitor. The column was developed by a gradient of pyridine–acetic acid buffers (for details see the text). Peptide elution was continuously monitored by automatic ninhydrin analysis after alkaline hydrolysis.

Table III: Amino Acid Compositions (Residues per Molecule) of Peptides Derived from Carboxypeptidase Inhibition by Cleavage of the Asp_5–Pro_6 Bond.

	Residues 1–5		Residues 6–39	
Amino Acid[a]	Predicted[b]	Found	Predicted[b]	Found
Lys			2	2.5
His	1.0	1.0	1	0.9
Arg			1	1.2
Asp	1.0	1.0	4	3.7
Thr			2	1.7
Ser			2	1.7
Glu	1.5	1.6	1	0.9
Pro			3	2.6
Gly			3	2.7
Ala	1.0	1.0	3	3.0
Cys			6	6.7
Val			1	0.8
Ile			1	0.6
Tyr			1	0.9
Phe			1	0.9
Trp			2	N.D.[c]

[a] All other amino acids present at less than 0.2 residue per molecule. [b] Taken from the amino acid sequence (Hass et al., 1975). [c] Not determined.

(Table I) suggested that this derivative containing modified arginine-32 bound enzyme as tightly as the unmodified inhibitor. In view of both the change in charge and the bulk attending modification of arginine by phenylglyoxal, it is unlikely that arginine-32 is in contact with enzyme in the complex.

Fragment AP-2. Aspartyl–proline bonds are particularly susceptible to acid hydrolysis and may be selectively cleaved (Fraser et al., 1972). Peptide fragments derived by specific acid cleavage of the Asp_5–Pro_6 bond of the *S*-carboxymethyl and *S*-pyridylethyl derivatives of the inhibitor were employed in amino acid sequence analysis (Hass et al., 1975). In order to evaluate the importance of residues 1–5 to the enzyme–inhibitor interaction, fragment AP-2 (residues 6–39) was prepared by acid treatment in 7 *M* guanidine hydrochloride. After desalting the peptides by gel filtration on Sephadex G-25 F, the peptides AP-1 and AP-2 were separated by ion-exchange chromatography on SE-Sephadex C-25 (Figure 5). Fractions denoted as SE-1 and SE-2 were identified by amino acid analysis as trace amounts of intact inhibitor and as the mixture of amino terminal peptides, respectively (Table III).

Fraction SE-3 was identified by amino acid composition as fragment AP-2 (residues 6–39) (Table III). In addition, automatic Edman degradation in the Beckman sequencer confirmed identification of pool SE-3 as AP-2. The binding of AP-2 to both carboxypeptidases A and B was approximately as strong as that of the unmodified inhibitor (Table I) suggesting that residues 1–5 do not participate in binding to the target enzymes.

N^{ϵ}-Acetyllysine Inhibitor. Acetylation of the inhibitor in 50% saturated sodium acetate with acetic anhydride modified only the ϵ-amino groups of lysine residues 10 and 13, since the α-amino group was blocked and high concentrations of acetate catalyzed the hydrolysis of *O*-acetyltyrosine residues (Riordan and Vallee, 1967). After two treatments with acetic anhydride, modification was essentially complete since titration of the *N*-acetyl inhibitor with 2,4,6-trinitrobenzenesulfonate indicated an average of only 0.05 residue amino groups remaining per molecule. The *N*-acetyl derivative bound to carboxypeptidases A and B as strongly as native inhibitor (Table I) implying that lysine residues 10 and 13 do not participate in binding.

Tryptophan Modification. Treatment of the inhibitor with *o*-nitrophenylsulfenyl chloride (Scoffone et al., 1968) in 80% acetic acid for 1 hr modified an average of only 0.15 tryptophan residue of a possible two residues (positions 22 and 28). Under identical conditions an average of 1.2 residues of tryptophan per molecule of *S*-Cm inhibitor were modified. In addition, 2-hydroxy-5-nitrobenzyl bromide (Horton and Koshland, 1965) at neutral pH modified little (0.23 residue per molecule) of these tryptophan residues in native inhibitor. These data suggest that the two tryptophan residues are buried in the interior of the molecule and are relatively inaccessible to solvent. Thus, these residues presumably do not come in contact with enzyme in the complex.

Discussion

Proteinaceous inhibitors of the "serine" proteases are found in a variety of plant and animal tissues (for review see Laskowski and Sealock, 1971). To date, however, naturally occurring inhibitors of the pancreatic carboxypeptidases have only been found in potatoes (Ryan et al., 1974) and in *Ascaris lumbricoides* (Homandberg and Peanasky, 1974). The recent elucidation of the amino acid sequence of the inhibitor from potatoes (Hass et al., 1975), its small size, extreme stability, relative ease of preparation, and tight binding with target enzymes (Ryan et al., 1974) have prompted further investigation of the enzyme–inhibitor interactions.

This report attempts to identify the contact region of the carboxypeptidase inhibitor from the effects of several chemical modifications on the strength of its binding to carboxypeptidases A and B. K_I values for the inhibitor and its derivatives have been determined either by the method of Lineweaver and Burk (1934) or of Henderson (1972, 1973). The latter procedure, which considers the depletion of inhibitor upon binding to enzyme, provides better estimates of

K_I when the inhibitor to enzyme ratio is relatively low. Although such low mole ratios are necessary when the inhibition of carboxypeptidase A is investigated (Ryan et al., 1974), the similarity of K_I values for native inhibitor determined by Henderson's method and by Lineweaver–Burk analysis (Table I) suggests that either procedure may be used effectively.

An increase in K_I value attending a given modification (e.g., attachment of a leucyl residue to the α carboxylate of glycine-39) suggests that the unaltered residue is in the contact region in the enzyme–inhibitor complex. However, the possibility that the modification merely induces a deleterious conformational change in the inhibitor must not be excluded. Similarly, the failure of a modification to alter K_I values suggests that the modified residue does not contribute significantly to the free energy of binding, but does not preclude its proximity to the enzyme in the complex.

With these limitations on interpretation in mind, a systematic series of modifications of the carboxypeptidase inhibitor was undertaken which would allow tentative identification of regions of the inhibitor in contact with enzyme and would provide support for a single site model of inhibition of the carboxypeptidases. The relevant data are summarized in Figure 1 and Table I.

The most informative series of experiments involved the tetraleucyl inhibitor. This derivative was prepared by coupling leucine to the α carboxylate of the inhibitor and to the β carboxylates of aspartic acid residues 5, 16, and 17. It possesses little inhibitor activity. Selective removal of leucine from the carboxyl-terminal position of this derivative regenerates inhibitory activity. This suggests that the α carboxylate of glycine-39 participates in the enzyme–inhibitor interaction and that the three β carboxylates are not critically involved. The participation of a carboxylate in binding to enzyme might be anticipated since carboxypeptidases require an α carboxylate on substrates (Pétra, 1970).

Tyrosine-37 is also a part of the contact region between enzyme and inhibitor. Although it may be acetylated with little effect on inhibitory activity (Table I), this residue is believed to be shielded by enzyme since carboxypeptidases A and B protect the *O*-acetyltyrosine derivative from hydroxylamine-induced deacetylation. In preliminary studies (Hass et al., 1974) tyrosine-37 has been implicated in enzyme–inhibitor interaction based upon relatively weak binding of the nitro derivative of the inhibitor. However, it has been subsequently shown in this laboratory[2] that, during nitration, the inhibitor becomes partially cross-linked and some destruction of tryptophan occurs.

Modification of other residues had little effect on the strength of binding of the inhibitor to carboxypeptidases A and B with the possible exception of the histidine residues (Table I). However, the relatively small change attending the introduction of two carboxamidomethyl groups per histidine residue suggests a minor contribution of these residues to the free energy of binding.

Derivatives of the inhibitor in which arginine-32 is modified by treatment with phenylglyoxal, lysine residues 10 and 13 are acetylated, or residues 1–5 are removed by specific acid cleavage are each as potent as the native inhibitor. These residues, the buried tryptophan in positions 22 and 28, aspartic acid residues 5, 16, and 17, tyrosine-37, and histidine residues 5 and 16 comprise a rather substantial part (14 of 39 residues) of the inhibitor which presumably contributes little to the binding energy of 12 kcal/mol (Figure 1). These studies therefore suggest that the carboxyl-terminal sequence Tyr-Val-Gly-OH is a part of the contact region of the inhibitor.

It is unlikely that the energy of binding between enzyme and inhibitor is due merely to the linear sequence of a few amino acid residues which complement a region on the enzyme since model peptide substrates normally bind weakly to a carboxypeptidase A with K_m values greater than 1×10^{-4} *M* (Pétra, 1970). To demonstrate the requirement of the structural integrity of the inhibitor for inhibitory activity, it has been reduced and *S*-carboxymethylated. This derivative is not inhibitory at the concentrations tested.

As pointed out by Blow (1974), model peptide substrates or inhibitors of proteolytic enzymes must lose translational and rotational degrees of freedom when bound to their target enzymes, resulting in an unfavorable entropy term in the binding constant. However, a conformationally restrained sequence of amino acids such as occurs in proteinaceous protease inhibitors can bind quite tightly to a protease because entropy loss is minimized. In the present work, a comparison of peptide T-2 to native inhibitor may be an example of this principle. Peptide T-2, the carboxyl-terminal hexapeptide (residues 33–39), contains Tyr-37 and the essential Gly-39 but binds to carboxypeptidase A poorly ($K_I > 4 \times 10^{-4}$ *M*) compared with native inhibitor ($K_I = 5.2 \times 10^{-9}$ *M*). The requirement of the conformational integrity of this inhibitor is analogous to the requirement of the conformational integrity of protein inhibitors of serine proteases (Sweet et al., 1974).

These studies of the effects of chemical modification of the carboxypeptidase inhibitor on its inhibitory activity not only provide an indication of those residues which most likely are in contact with the enzyme in the complex, but also suggest that a large part of the inhibitor (14 of 39 residues) does not make contact with enzyme. This also is analogous to the mechanism of action of some serine protease inhibitors. Crystal structure analysis of the Kunitz soybean and pancreatic trypsin inhibitors (Rühlmann et al., 1973; Sweet et al., 1974) demonstrate that only a small region of the proteinase inhibitor interacts directly with the enzyme.

The parallel effects of each modification on the strength of binding to carboxypeptidases A and B support data from competition experiments (Ryan, 1971) which indicate that both enzymes bind to the same inhibitor site. The identification of the carboxyl-terminal amino acid (glycine-39) as critical for enzyme–inhibitor interaction also favors a single site model. In view of the similar binding constants of the inhibitor for carboxypeptidases A and B (Table I), it might be anticipated that the reactive site of the inhibitor contains neither a hydrophobic nor a basic residue which, if protruding into the active site of the enzyme in the complex, would generate a preference for carboxypeptidase A or carboxypeptidase B, respectively.

The mechanism of inhibition has been probed in a parallel study by investigating the binding of the inhibitor to derivatives of carboxypeptidase A (H. Ako et al., in preparation). The strong binding to carboxypeptidase A in which the enzyme's binding site and portions of its active site region are blocked by an affinity label (Hass and Neurath, 1971) suggests that the binding of inhibitor does not directly involve that part of the active site occupied by the affinity label. This is in marked contrast to most "serine" protease–protein inhibitor interactions (Laskowski and Sealock,

[2] T. R. Leary, unpublished observations.

1971) which require a vacant binding pocket. The similarity of the spectral changes attending binding of the inhibitor to the arsanilazotyrosine derivative of carboxypeptidase A to those induced by substrates, however, suggests that the inhibitor is bound near the active site (Ako, H., Hass, G. M., Grahn, D. T., and Neurath, H., in preparation; Hass et al., 1974). To explain both observations, a model has been proposed in which the inhibitor fits like a cap over the active site region. Its strength of binding to carboxypeptidases A and B is derived from contacts between homologous regions on the enzymes with a complementary region on the inhibitor.

The data obtained in these studies provide a partial understanding of the mechanism of action of the carboxypeptidase inhibitor which should complement the three-dimensional structure of the inhibitor or enzyme-inhibitor complex when it becomes elucidated. With information from both x-ray crystallographic analysis and solution chemistry at hand, a reasonably accurate estimation of the contributions of certain interactions to the overall binding energy may be possible (Huber et al., 1970; Janin et al., 1974).

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