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INTERACTION OF CARBOXYPEPTIDASES A AND B WITH NITROTYROSYL AND AMINOTYROSYL DERIVATIVES OF THE CARBOXYPEPTIDASE INHIBITOR FROM POTATOES

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Key Word Index—*Solanum tuberosum*; Solanaceae; potato; carboxypeptidase inhibitor; nitration; chemical modification; protease inhibitor.

Abstract—The single tyrosine residue of the carboxypeptidase inhibitor from potatoes, which is in contact with carboxypeptidase A in the enzyme–inhibitor complex as determined by X-ray diffraction, was converted to 3-nitrotyrosine by treatment with tetranitromethane in buffers containing 75% ethanol. The nitroinhibitor bound both bovine carboxypeptidase A and porcine carboxypeptidase B with apparent K_i values indistinguishable from those of the unmodified inhibitor. Spectral titration indicated that the nitrotyrosyl residue of the inhibitor ionized with a pK_a of 7.25 either in the presence or absence of carboxypeptidase A; however, this pK_a was shifted to *ca* 7.7 in the presence of carboxypeptidase B. Reduction of the 3-nitrotyrosine residue to 3-aminotyrosine slightly increased the strength of binding to both carboxypeptidases. These data suggest that the tyrosine residue of the inhibitor is in a polar environment in the enzyme–inhibitor complex and that it is not involved in hydrogen bonding.

INTRODUCTION

The carboxypeptidase inhibitor from potatoes, a mixture of polypeptides of 38 and 39 amino residues, competitively inhibits the pancreatic carboxypeptidases with K_i values of 5–50 nM [1]. Earlier studies of the interactions of several derivatives of the inhibitor with target enzymes suggested that carboxypeptidases A and B bound the inhibitor in a similar fashion and that the carboxyl-terminal region of the inhibitor was involved in the binding process [2]. Although the single tyrosine residue of the inhibitor (position 37) could be acetylated without affecting the strength of binding to target enzymes, the enzyme prevents deacetylation of this derivative by hydroxylamine. These data suggested that tyrosine-37 was in the contact zone in the enzyme–inhibitor complex [2].

Recently, the structure of the carboxypeptidase inhibitor–carboxypeptidase A complex at 0.25 nm resolution has been reported [3]. The ring of tyrosine-37 protrudes between the side chains of threonine-164 and tyrosine-248 of the enzyme. This report describes the use of the 3-nitrotyrosyl and 3-aminotyrosyl derivatives of the inhibitor to elucidate further the environment of tyrosine-37 in the complex and to afford a means of comparing the enzyme–inhibitor complex in solution and in the crystalline state.

RESULTS

Preparation and characterization of inhibitor derivatives

Tyrosine-37 was nitrated by incubating the inhibitor for 4 hr with a 50-fold molar excess of tetranitromethane in buffers containing ethanol [4]. Purification of the nitroinhibitor was accomplished by gel filtration and ion exchange chromatography. The 3-aminotyrosyl derivative of the inhibitor was rapidly formed from the nitroinhibitor by reduction with sodium hydrosulfite [5]. After ion exchange chromatography on carboxymethylcellulose, this derivative contained an average of 0.9 residues of 3-aminotyrosine per molecule with no evidence of 3-nitrotyrosine.

Apparent K_i *values.* Apparent K_i values for the inhibition of bovine carboxypeptidase A and of porcine carboxypeptidase B by unmodified inhibitor, nitroinhibitor and the aminotyrosyl derivative (Table 1) were determined at pH 7.5 using the method of Green and Work [6]. The nitration of the inhibitor had no appreciable effect on the strength of binding to either enzyme. However, subsequent reduction of the nitro group to an amino substituent slightly decreased the apparent K_i with respect to both carboxypeptidase A and carboxypeptidase B.

Spectral titration. The pK_a value exhibited by the nitrotyrosine of the modified inhibitor was determined by spectral titration at 428 nm (Fig. 1A). The apparent pK_a of 7.25 ± 0.05 was similar to those of nitrophenol groups in a variety of model compounds, pK_a 6.8–7.2 [7]. Spectral titration of nitroinhibitor in the presence of excess carboxypeptidase A was indistinguishable from that of inhibitor alone (Fig. 1A). Essentially all of the modified inhibitor was complexed with enzyme in this study, since (a) K_i values for nitroinhibitor determined at pH 6.5, 7.5 and 8.5 were small (*ca* 10 nM) compared with the concentrations of enzymes and inhibitor in the spectral titration experiments, and (b) an excess of carboxypeptidase A was used.

The pK_a of the 3-nitrotyrosine group in the nitroinhibitor–carboxypeptidase B complex was 7.7 ± 0.1 as determined by spectral titration (Fig. 1B). This relatively small shift in pK_a, relative to free nitroinhibitor, was reproduced in several experiments.

Table 1. Inhibition of carboxypeptidases by nitrotyrosyl and aminotyrosyl derivatives of the carboxypeptidase inhibitor from potatoes

Derivative	Carboxypeptidase A $K_{i,app}$ (nM)*	Carboxypeptidase B $K_{i,app}$ (nM)
Unmodified	1.5–2.6†	5–18
Nitrotyrosyl	1.1–2.6	7–12
Aminotyrosyl	0.3–1.0	3–4

*Determined by the method of ref. [6] using hippuryl-L-phenylalanine as substrate for carboxypeptidase A and hippuryl-L-lysine for carboxypeptidase B. Apparent K_i values are related to true K_i values by the equation $K_{i,app} = K_i\left(1 + \frac{[S]}{K_s}\right)$ where [S] is the substrate concentration and K_s the Michaelis constant.

†The range of $K_{i,app}$ values represents three determinations each.

DISCUSSION

The carboxypeptidase inhibitor used in these studies was a mixture of polypeptides of 38 and 39 amino acid residues differing only in their amino-terminal regions [8]. In earlier studies, the carboxyl-terminal region of the inhibitor was implicated in binding to target enzymes, since addition of leucine to alpha-carboxylate of glycine-39 prevented binding to both carboxypeptidases A and B, and since carboxypeptidase A protected against deacetylation of the single tyrosine residue (position 37) [2]. A recent report describing the structure of the carboxypeptidase A–carboxypeptidase inhibitor complex has confirmed the involvement of the carboxyl-terminal region of the inhibitor in binding to enzyme and identified other contact points as well [3]. Both the peptide backbone surrounding tyrosine-37 and the ring of this residue were identified as interacting with the enzyme.

Nitration of the inhibitor was selected to investigate further the role of tyrosine-37 in enzyme–inhibitor interactions for several reasons; (a) the modification reaction occurs under mild conditions and is reasonably specific for tyrosine [9]; (b) the nitro group increases the bulk of the tyrosine moiety allowing investigation of steric constraints on binding; (c) 3-nitrotyrosine has a pK_a which can readily be determined and which occurs in a pH region where carboxypeptidase–carboxypeptidase inhibitor interactions are strong; (d) the nitro group can be readily reduced to the amino function.

$K_{i,app}$ determinations (Table 1) indicated that addition of the rather bulky nitro group to the ring of tyrosine-37 did not appreciably affect the strength of binding of the inhibitor to either carboxypeptidase A or B. Thus, the contact area about the ring of tyrosine-37 could not be particularly confined, a finding consistent with the tight binding of the *O*-acetyltyrosyl derivative of the inhibitor to carboxypeptidases A and B [2]. The enzyme–inhibitor interaction was unaffected by a negative charge on the phenolic hydroxyl of tyrosine-37, since this residue is partially ionized in the enzyme–nitroinhibitor complexes (Fig. 1). The aminophenol derivative bound both carboxypeptidases with slightly lower K_i values than those of the unmodified inhibitor (Table 1). These effects were probably not due to ionic interactions because the amino and phenol groups, which have pK_a values of *ca* 5 and 10, respectively, were not ionized under these assay conditions [5].

The apparent pK_a of the nitrotyrosine in the modified inhibitor was 7.25 (Fig. 1), a value on the high end of the pK_a range of a series of model compounds studied previously [7]. These data suggested that tyrosine-37 might participate in hydrogen bonding, be in a slightly hydrophobic region of the inhibitor, or be near a negatively charged residue. The presence of carboxypeptidase A did not alter the pK_a of the nitrotyrosine, whereas a shift in pK_a was observed when carboxypeptidase B was bound (Fig. 1). This shift in pK_a of 0.5 pH units which attended the binding of carboxypeptidase B to the nitroinhibitor was small compared with an increase in pK_a of at least 4 pH units when nitrated soybean trypsin inhibitor (Kunitz) bound to trypsin [10]. The latter shift in pK_a was attributed both to burying the nitrotyrosine in a hydrophobic environment and to formation of a hydrogen bond in the trypsin–inhibitor complex [10].

With the $K_{i,app}$ values of the nitrotyrosyl and aminotyrosyl derivatives of the inhibitor for the carboxypeptidases (Table 1) and the pK_a of values for 3-nitrotyrosine-37 in enzyme–inhibitor complexes (Fig. 1) in mind, it was of interest to observe the environment of tyrosine-37 as deduced by X-ray diffraction of the inhibitor–carboxypeptidase A complex [3]. The ring of tyrosine-37 was said to be directed between the side chains of threonine-164 and tyrosine-248 of the enzyme with tyrosine-248 in the down position [3]. Both threonine-164 and tyrosine-248 have been conserved in the carboxypeptidase B molecule as have most of the other amino acid residues in this immediate vicinity (arginine-127, serine-162, and glutamic acid-163) [11]. Thus, it was not surprising that carboxypeptidases A and B responded in a parallel fashion to modification of tyrosine-37 of the inhibitor (Table 1). From the decidedly hydrophilic nature of the contact zone, little shift in the pK_a of nitrotyrosine-37 of the inhibitor would be predicted. Unfortunately, further refinement of the structure of the enzyme–inhibitor complex or a more detailed description of the available information is required to understand the modest pK_a shift of nitrotyrosine-37 of the inhibitor attending binding of carboxypeptidase B (Fig. 1B). Similarly, it is impossible at this time to determine whether the structure proposed for the enzyme–inhibitor complex will easily accommodate the introduction of a 3-nitro or *O*-acetyl [2] group on tyrosine-37 of the inhibitor.

EXPERIMENTAL

Materials. The carboxypeptidase inhibitor was purified from Russet Burbank potatoes by the method of ref. [1]. Bovine carboxypeptidase A was purchased from Worthington Biochemical Corporation and porcine carboxypeptidase B was prepared from trypsin-activated pancreatic extracts [12]. Hippuryl-L-phenylalanine and hippuryl-L-arginine were from Sigma, hippuryl-L-lysine was from Vega-Fox and tetranitromethane was purchased from Aldrich.

Preparation and characterization of inhibitor derivatives. 3-Nitrotyrosyl carboxypeptidase inhibitor was routinely prepared as described previously [4]. 3-Aminotyrosyl inhibitor was prepared by reduction of the nitro-derivative with sodium hydrosulfite ($Na_2S_2O_4$) [5]. An 8-fold molar excess of $Na_2S_2O_4$ was added to a soln of nitroinhibitor (0.22 mM) in 3 ml 0.5 M NaCl, 5 mM Tris–HOAc (pH 8). The reduction was

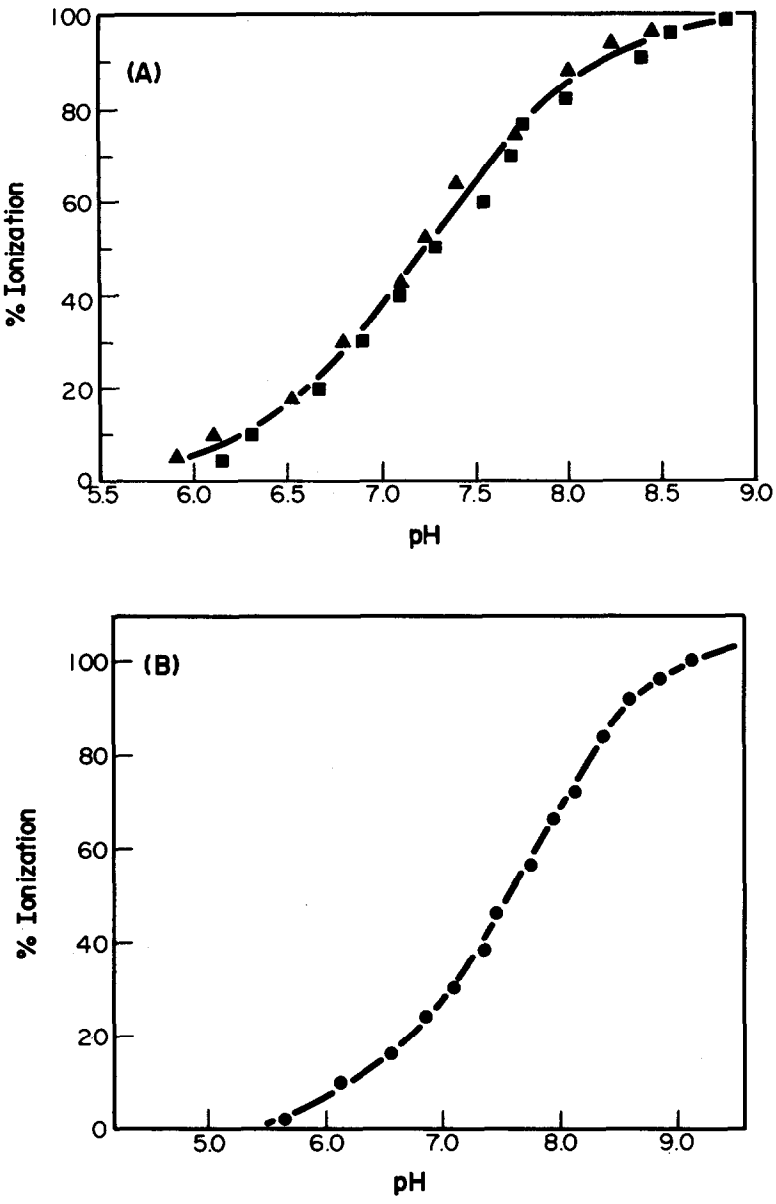

Fig. 1. Ionization of nitrotyrosine-37 of modified carboxypeptidase inhibitor as a function of pH. Spectral titrations of the free nitroinhibitor (■——■) and the nitroinhibitor–carboxypeptidase A complex (▲——▲) (A) and of the nitroinhibitor–carboxypeptidase B complex (●——●) (B) were performed as described in Experimental.

complete within 5 min as evidenced by the absence of a further decrease in A_{428} nm. The sample was dialysed against 10 mM Na citrate (pH 4.3) and chromatographed on carboxymethylcellulose [1, 2]. Amino acid analyses of the inhibitor and its 3-aminotyrosyl and 3-nitrotyrosyl derivatives were performed as described previously [1]. Tryptophan was determined on hydrolysates prepared in 4 N methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole [13].

Estimation of $K_{i,app}$. Apparent K_i values for the inhibition of the carboxypeptidases were obtained at 25° by titration of enzymatic activity [6]. Bovine carboxypeptidase A (final concn typically 0.1 μM) was assayed spectrophotometrically using 3.1 ml assay solns containing 1 mM hippuryl-L-phenylalanine, 0.5 M NaCl, 50 mM Tris–HOAc, and varying amounts of inhibitor [14]. Although the assay solns were usually pH 7.5, certain expts were performed at pH values of 6.5 and 8.5. Porcine carboxypeptidase B was assayed spectrophotometrically at 254 nm using 1 mM hippuryl-L-arginine or 1 mM hippuryl-L-lysine in 0.5 M NaCl, 50 mM Tris–HCl (pH 7.5) [15]. Enzyme concns in the 3.1 ml assay solns were *ca* 27 μM using hippuryl-L-arginine and 0.14 μM with hippuryl-L-lysine.

Spectral titrations of nitroinhibitor and of nitroinhibitor–carboxypeptidase complexes. Nitroinhibitor was dissolved in 0.5 M NaCl, 5 mM Tris–HOAc (pH 6) and small

amounts of NaOH or HCl (0.2 M) were added with stirring. After each addition the pH and $A_{428\,nm}$ were determined. Concns of nitroinhibitor used in these expts (*ca* 0.2 mM) were estimated from the $E_{428\,nm} = 4100/M/cm$ for the nitrophenolate ion and by titration with carboxypeptidase A. Spectral titrations of the nitroinhibitor–carboxypeptidase complexes were performed as above except that titration was only from low to high pH, since the addition of even small amounts of HCl produced some protein precipitation. Typical nitroinhibitor and enzyme concns employed in these studies were 0.1 and 0.15 mM, respectively.

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