Chemical Modification of Carboxypeptidase A Crystals. Nitration of Tyrosine-248†

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ABSTRACT: Nitration of bovine carboxypeptidase A crystals with tetranitromethane increases esterase activity, decreases peptidase activity, and modifies about one tyrosyl residue. Modification of enzyme crystals avoids the polymerization that occurs when the enzyme is nitrated in solution. Two procedures have been employed to identify the tyrosyl residues nitrated. The first involves cyanogen bromide cleavage and isolation of the fragment containing residues 104–301. After solubilization by succinylation, this fragment is digested with chymotrypsin, the peptides are fractionated by gel filtration, and the nitrotyrosyl peptides are purified by affinity chromatography on an antinitrotyrosyl antibody-Sepharose conjugate followed by ion-exchange chromatography. In the second, the nitroenzyme is heat denatured, digested by chymotrypsin, and fractionated on the affinity and ion-exchange columns. By both methods, the major nitropeptides, representing between 60 and 80% of the nitrotyrosyl label, are uniquely compatible with that segment of the sequence of carboxypeptidase containing Tyr-248. A nearby cation, either the active site zinc ion or Arg-145, would seem to be an important factor in determining the selective nitration of this residue.

Acetylation with *N*-acetylimidazole (Simpson et al., 1963) and acetic anhydride (Riordan and Vallee, 1963) first indicated that tyrosyl residues play a role in the catalytic mechanism of carboxypeptidase A. These reagents reduce the peptidase activity of the enzyme and increase its esterase activity sixfold. The inhibitor, β-phenylpropionate, prevents these activity changes and protects two tyrosyl residues against *O*-acetylation. The changes in activities are reversible on exposure to neutral hydroxylamine; two *O*-acetyltyrosyl groups of the modified enzyme deacylate faster than any of the others. Based on acetylation alone, it could not be determined whether these tyrosyl residues have the same or different functions, or whether just one is important in the catalytic process, the other reacting coincidentally.

Similar changes in esterase and peptidase activity also occur on nitration of only one tyrosyl residue of carboxypeptidase A (Riordan et al., 1967). Prior acetylation prevents the functional effects of nitration and vice versa suggesting that the tyrosyl residue nitrated by tetranitromethane is one of those acetylated by either acetic anhydride or *N*-acetylimidazole.

The 2.0-Å resolution structure of carboxypeptidase A and its complex with glycyl-L-tyrosine (Lipscomb et al., 1968) together with the sequence information of Bradshaw et al. (1969) provided evidence that Tyr-248 might be an essential component of the active site of the enzyme. It was postulated that this could be the residue modified by acetylation and nitration (Quiocho and Lipscomb, 1971). Johansen and Vallee (1971) modified carboxypeptidase with diazotized arsanilic acid and obtained a derivative containing a single arsanilazotyrosyl residue per molecule, afterwards identified as Tyr-248 (Johansen et al., 1972). However, the activities of the azo derivative are only slightly different from those of the native enzyme. In order to determine if these reagents modify the same tyrosyl residue, we have undertaken identification of the specific tyrosyl residue nitrated by tetranitromethane. Using affinity chromatography on an antinitrotyrosyl antibody column (Helman and Givol, 1971), we have isolated a number of nitrotyrosyl peptides in an overall yield of about 55%. Composition and amino terminus information demonstrates that approximately 80% of the nitro label is on Tyr-248. The identity of other tyrosyl residues, nitrated to fractional degrees, and their location in the tertiary structure provide a chemical basis for the selectivity of tyrosine modification in this protein. A preliminary account of these results has been presented (Muszynska and Riordan, 1973).

Materials

Carboxypeptidase A crystals (Worthington Biochemical Corp.) prepared according to Anson (1937) were washed three times with metal-free, distilled water, dissolved in 0.05 *M* Tris–2 *M* NaCl (pH 7.5) (40 mg/ml), and centrifuged at 40000 rpm for 90 min at 4° in a Spinco Model L ultracentrifuge. The supernatant was dialyzed against 0.05 *M* Tris (pH 7.5) at 4° and after 24 hr the resultant crystals were collected by centrifugation.

Chymotrypsin and ovalbumin were also obtained from Worthington and bovine serum albumin from Armour Pharmaceutical Corp. Tetranitromethane and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride were from Aldrich, 5-dimethylaminonaphthyl-1-sulfonyl chloride (dansyl chloride) and dansyl amino acids were from Pierce, micropolyamide sheets were from Schleicher and Schuell, agarose was from Fisher Scientific, Bio-Gel P-4 was from Bio-Rad Laboratories, DE-52 pre-swollen ion exchange cellulose and Whatman No. 3 MM chromatographic paper were from Reeve-Angel, and Sepharose 4B, DEAE-Sephadex A-25, and Sephadexes G-10 and G-75 were from Pharmacia Fine Chemicals, Inc. Propionic acid and pyridine, obtained from Fisher Scientific, were distilled prior to use and

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3-nitrotyrosine (Fox Chemical) was recrystallized from water. All other chemicals were of reagent grade and were used without further purification.

Methods and Results

Analytical Procedures. Amino acid analyses (Spinco Model 120C) were carried out on samples hydrolyzed with 6 *N* HCl in sealed evacuated tubes at 110° for 18–24 hr. The values reported are uncorrected. The ninhydrin color yield of 3-nitrotyrosine is identical with that for valine (Giese and Riordan, 1975). The concentration of carboxypeptidase solutions was determined from absorbance at 278 nm (ϵ_{278} 6.42 × 10^4 M^{-1} cm^{-1}). Nitrocarboxypeptidase concentration was determined similarly except the absorbance of the protein at 428 nm was first subtracted from that at 278 nm to correct for the contribution of nitration to absorbance. Nitropeptide concentrations were determined from absorbance measured at 381 nm, the isosbestic point for nitrotyrosine, ϵ_{381} 2200, or at 360 nm (ϵ_{360} 2790) for acidic samples and at 428 nm (ϵ_{428} 4200) for alkaline samples (Sokolovsky et al., 1966). A Zeiss PMQ II spectrophotometer was used for measurements of absorbance at single wavelengths and a Cary 14 RS spectrophotometer was employed for recording absorption spectra. Peptidase and esterase activities were determined with 0.02 *M* benzyloxycarbonylglycyl-L-phenylalanine in 0.05 *M* Tris–0.8 *M* NaCl (pH 7.5) and with 5 *M* hippuryl L-phenyllactate in 5 m*M* Tris–0.2 *M* NaCl (pH 7.5), respectively (Davies et al., 1968). N-terminal analyses were performed with dansyl chloride (Gray, 1967). Dansyl amino acids were identified by thin-layer chromatography on polyamide sheets (Woods and Wang, 1967).

Nitration of Carboxypeptidase. Procedures reported previously for nitration of carboxypeptidase (Riordan et al., 1967) were carried out with the enzyme (5 mg/ml) dissolved in 0.05 *M* Tris–1 *M* NaCl (pH 8.0) and using 0.6 m*M* tetranitromethane for 30 min at 20°. Approximately one tyrosyl residue is nitrated under these conditions, but the nitroenzyme is polymerized extensively. Such preparations exhibit a wide range of high molecular weight components on gel filtration. We have now carried out the nitration by addition of tetranitromethane, freshly diluted 1:50 into 95% ethanol—final concentration, 0.45 m*M*—to a suspension of carboxypeptidase crystals, 5 mg/ml, in 0.05 *M* Tris (pH 8.0) 20°, stirred gently, and kept in the dark. The reaction is stopped after 45 min by centrifuging and washing the crystals with the same buffer. Again approximately one tyrosyl residue is nitrated but in contrast to the solution procedure, enzyme nitrated in the crystalline state and then dissolved is monodisperse and elutes from a G-75 Sephadex column in the same position as native carboxypeptidase.

Antigens and Antibodies. Nitrotyrosyl bovine serum albumin and ovalbumin were prepared by coupling 3-nitrotyrosine to the proteins using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide according to the procedures of Helman and Givol (1971). A total of 23 nitrotyrosyl residues were incorporated per molecule of albumin and 10 per molecule of ovalbumin.

Antisera were raised by injecting white male New Zealand rabbits, in four locations, footpads and intramuscularly, with a total of 5 mg of 3-nitrotyrosyl albumin in 0.5 ml of 0.9% NaCl emulsified 1:1 (v/v) with complete Freund's adjuvant. Immunization with 2 mg of antigen, similarly diluted and emulsified, was repeated after 4, 8, 12, and 16 weeks, respectively. The animals were bled after 7, 13, and 17 weeks, and the sera from all bleedings were pooled. Precipitating antibodies were identified with nitrotyrosyl ovalbumin by immunodiffusion. Antibodies were isolated by two successive fractionations with 50% saturated $(NH_4)_2SO_4$ at room temperature. The precipitate was dissolved in 5 ml of 0.01 *M* phosphate–0.14 *M* NaCl (pH 7.4) and dialyzed against 0.01 *M* phosphate–0.14 *M* NaCl (pH 7.4) at 4° overnight. After removing a small amount of precipitate by centrifugation, the γ-globulin fraction was separated by ion-exchange chromatography on a DE-52-cellulose column. Fractions containing the antinitrotyrosyl ovalbumin activity were collected and dialyzed against 0.1 *M* $NaHCO_3$ (pH 8.0) for 8 hr at 4°. The concentration of the antibody solution was determined from absorbance at 280 nm using a factor of 1.4 for 1 mg/ml.

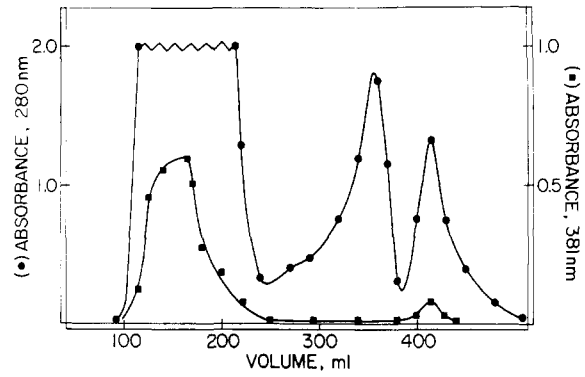

FIGURE 1: Separation of cyanogen bromide fragments of nitrocarboxypeptidase on G-75 Sephadex. A 500-mg sample of CNBr-treated nitrocarboxypeptidase prepared according to Nomoto et al. (1969) was applied to a 2.9 × 90 cm column of Sephadex G-75 equilibrated and developed with 0.1 *M* propionic acid; the flow rate was 36 ml/hr. Fractions of 3 ml were collected and absorbance was measured at 278 (●) and 381 nm (■).

Preparation of Modified Sepharose for Affinity Chromatography. Activation of Sepharose 4B with cyanogen bromide and covalent coupling of the antinitrotyrosyl albumin antibodies to the activated Sepharose were performed as described by Wilchek et al. (1971). The Sepharose conjugate was equilibrated with 0.01 *M* phosphate–0.14 *M* NaCl (pH 7.4) prior to use in separating nitrotyrosyl peptides.

Two methods have been employed to isolate nitrotyrosyl peptides from nitrocarboxypeptidase. The first involves more steps than the second but will be detailed because the peptides finally isolated provide important sequence information.

Method I. Cleavage of Nitrocarboxypeptidase A with Cyanogen Bromide and Separation of the Fragments. A typical preparation of nitrocarboxypeptidase (500 mg, 14.4 μmol) containing 1.1–1.2 nitrotyrosyl residues per molecule was cleaved with cyanogen bromide in 70% formic acid (Nomoto et al., 1969), and the fragments were separated on Sephadex G-75 equilibrated and developed with 0.1 *M* propionic acid (Figure 1). Greater than 95% of the nitrotyrosyl absorbance was present in F_I, i.e., residues 104–301, with the remainder in the F_N and F_C pool (residues 8–22[1] and 302–307, respectively). The amino acid composition of the F_I fragment agreed with that reported by Nomoto et al. (1969) and with the known sequence of the enzyme (Brad-

[1] Residues 1–7 are present in carboxypeptidase A_α but not in carboxypeptidase A_γ, the predominant species in enzyme preparations obtained from Worthington Biochemical Corp.

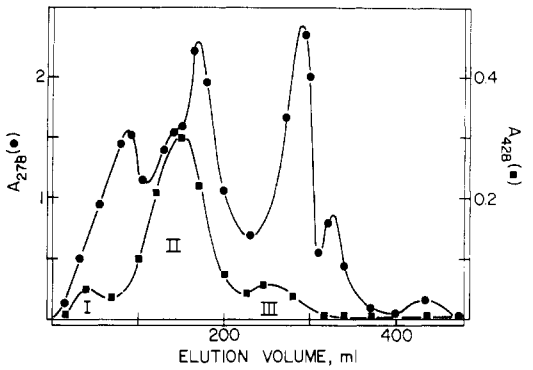

FIGURE 2: Separation of chymotryptic peptides on Bio-Gel P-4. *N*-Succinyl F_I was digested with chymotrypsin (Johansen et al., 1972) and the digest, 23 ml, applied to a 2.9 × 80 cm column of Bio-Gel P-4 equilibrated and developed with 0.5% NH_4HCO_3; the flow rate was 72 ml/hr. Fractions of 3 ml were collected and absorbance was measured at 278 (●) and 428 nm (■). The first 135 ml of eluate was discarded.

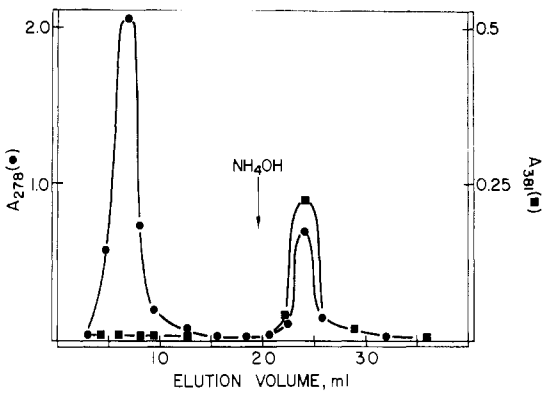

FIGURE 3: Isolation of nitrotyrosyl peptides by affinity chromatography. A sample, containing 0.25 μmol of nitrotyrosine from fraction II obtained by gel filtration of the chymotryptic digest of *N*-succinyl F_I (Figure 4), was applied to an antinitrotyrosyl antibody-Sepharose column (0.9 × 10 cm) equilibrated with 0.01 *M* NH_4HCO_3. After the main peak of absorbance at 278 nm was fully eluted, the nitrotyrosyl peptides were eluted with 1 *M* NH_4OH. Fractions of 1.6 ml were collected and absorbance was measured at 278 (●) and 381 nm (■).

shaw et al., 1969) except that approximately one residue of tyrosine was replaced by nitrotyrosine.

Succinylation of F_I, Desuccinylation, and Chymotrypsin Digestion. These were carried out as described previously for arsanilazocarboxypeptidase (Johansen et al., 1972). The yield of nitrotyrosine after these steps was 92%.

Isolation of Peptides from the Chymotrypsin Digest. The pH of the peptide mixture was adjusted to 8.0 and an aliquot containing 7.8 μmol of nitrotyrosine was applied to a Bio-Gel P-4 column equilibrated and developed with 0.5% NH_4HCO_3 (Figure 2). The recovery of absorbance was 98%. Three nitrotyrosine-containing fractions (i.e., A_{428}) were collected and lyophilized: I (15–90 ml), II (91–198 ml), and III (199–300 ml). The relative distribution of nitrotyrosine in the three fractions was 9:78:13.

Each lyophilized fraction was dissolved in 0.01 *M* NH_4HCO_3 (prepared fresh and adjusted to pH 7). An aliquot containing about 0.25 μmol of nitrotyrosine was applied to an antinitrotyrosyl antibody-Sepharose column equilibrated with the same buffer. The column was washed with buffer until the effluent absorbance at 278 nm returned to baseline. A typical elution profile is shown in Figure 3. Virtually all of the nitrotyrosyl absorbance remained on the column. This was eluted quantitatively with 1 *M* NH_4OH. The progression of a yellow band through the column served as a monitor of elution. The column was washed immediately with 0.01 *M* phosphate–0.14 *M* NaCl (pH 7.4) then with 0.01 *M* NH_4HCO_3 and the entire procedure repeated until all of the three fractions had been processed. The breakthrough fractions from each immunoaffinity chromatographic run were concentrated by lyophilization and rechromatographed in order to ensure maximal recovery of all nitrotyrosyl peptides. Nitrotyrosyl fractions I, II, and III were pooled separately and lyophilized.

Each fraction was subjected to further purification. Fraction I, which originally contained 9% of the A_{428} material eluting from the Bio-Gel P-4 column, was examined by high-voltage paper electrophoresis (vide infra). One major yellow band was detected. It contained approximately 100 amino acid residues per residue of nitrotyrosine and, based on its gel filtration elution characteristics, would appear to represent partially digested starting material. It was not examined further.

The nitropeptides from fractions II and III, after lyophilization, were dissolved in 0.1 *M* Tris-Cl (pH 8.3) and applied, in turn, to a DEAE-Sephadex A-25 column equilibrated with the same buffer. Elution with LiCl gradient gave two peaks of nitrotyrosyl absorption from fraction II (Figure 4). Fraction IIA (50–62 ml) contained 0.83 μmol (31%) of the applied sample and fraction IIB (103–122 ml) 0.83 μmol (29%) of nitrotyrosine. Both IIA and IIB were subsequently purified by high-voltage paper electrophoresis (vide infra).

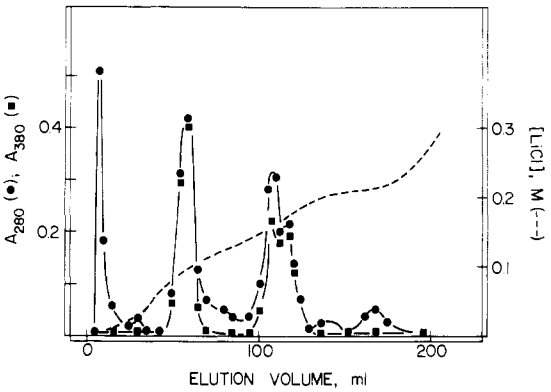

FIGURE 4: Chromatography of nitrotyrosyl peptides on DEAE-Sephadex A-25. The nitrotyrosyl peptides from fraction II, purified by affinity chromatography, were applied to a 0.9 × 8 cm column of DEAE-Sephadex A-25 equilibrated with 0.1 *M* Tris-Cl (pH 8.3). The column was developed at a flow rate of 10 ml/hr. A nine chamber gradient mixing device was connected to the column immediately after applying the sample. Chamber 1 contained 25 ml of 0.1 *M* Tris-Cl (pH 8.3) and 0.02 *M* LiCl. Chambers 2–9 were the same except that the LiCl concentrations were 0.075, 0.125, 0.15, 0.20, 0.15, 0.15, 0.20, and 0.50 *M*, respectively. Fractions of 2.4 ml were collected and absorbance was measured at 280 (●) and 380 nm (■).

The same chromatographic gradient was used for purification of fraction III. Three nitrotyrosine-containing peaks were separated and these were collected and lyophilized. Fraction IIIA contained 0.15 (23%), IIIB 0.12 (19%), and IIIC 0.37 μmol of nitrotyrosine (58% of the applied sample), respectively. The amino acid compositions of peptides IIIA and IIIB are given in Table I. Clearly, each arises from a different segment of the primary structure of carboxypeptidase A. The composition and N-terminal His, determined by dansylation (Gray, 1967) and subsequent thin-layer chromatography (Woods and Wang, 1967) of the re-

Table I: Amino Acid Composition of Nitrotyrosyl Peptides from a Chymotrypsin Digest of the Succinyl-F_1 CNBr Fragment.

Amino Acid	IIA-1	(241–248)[a]	IIA-2	(270–277)[a]	IIB-1	(239–248)[a]	IIB-2	(166–169)[a]	IIA	(166–169)[a]	IIIB	(241–248)[a]	IIIC-1	(239–243)[a]	IIIC-2	(246–248)[a]
Lys					1.1	1	0.8	1	0.9	1			0.8	1		
His							0.8	1	1.0	1						
Arg			2.0	2												
Asp			1.1	1												
Thr	2.0	2	1.1	1	2.1	2					2.2	2			1.0	1
Ser	1.0	1			1.2	1					1.1	1	0.8	1		
Glu			0.9	1												
Gly	1.1	1	1.1	1	1.3	1	0.9	1	1.0	1	1.2	1	1.1	1		
Ile	2.8	3			2.7	3					3.1	3	0.9	1	0.8	1
Leu			1.0	1												
Tyr		1		1	1.0	2		1		1		1		1		1
NO_2-Tyr	1.0		1.0		1.0		1.0		1.0		1.0		1.0		1.0	
N-Terminus	Gly		Gly		Lys		His		His		Gly		Lys		Thr	
Yield (%)[b]	17		7		13		9		3		3		4		4	
Relative yield (%)	28		12		22		15		5		5		7		7	

[a] From Bradshaw et al. (1969). [b] Fractions IIA, IIB, and IIIC were recovered in yields of 24, 22, and 8%, respectively. The yields of the various subfractions are estimated from the relative recoveries of material eluted from paper electropherograms.

sultant dansyl amino acid from peptide IIIA, are uniquely compatible with the sequence of residues 166–169 of carboxypeptidase (Bradshaw et al., 1969). The composition and N-terminal Gly of peptide IIIB identify its origin as residues 241–248.

Peptide fraction IIIC was a mixture of two nitrotyrosyl peptides which could be separated by paper electrophoresis. Compositions and N-termini indicate that peptides IIIC-1 and IIIC-2 originate from residues 239–243 and 246–248, respectively.

Desalting of Fractions IIA and IIB. These lyophilized fractions were dissolved in water and separately gel filtered through a 2 × 40 cm column of Sephadex G-10, equilibrated and developed with water at a flow rate of 40 ml/hr. The material eluting between 46 and 56 ml contained nitrotyrosine and was free of salt.

High-Voltage Paper Electrophoresis. After lyophilization the desalted fractions IIA and IIB were dissolved in water and 0.2 μmol of each was applied to Whatman No. 3MM paper. Electrophoresis was carried out in pyridine–acetic acid–water, 200:8:1800, pH 6.5, for 3 hr at 2 kV. The nitrotyrosyl peptides were visualized after separation by exposing the dried electropherogram to ammonia vapors.

Fraction IIA separates into two subfractions, IIA-1 and IIA-2. The former was found at the origin and could only be eluted from the paper with 0.5 *N* NaOH. The latter migrated toward the cathode and was eluted with 0.1 *N* HCl. Fraction IIB also separates into two subfractions. Again, IIB-1 was found at the origin, but IIB-2 migrated toward the anode. IIB-1 was eluted with 0.5 *N* NaOH and IIB-2 with 0.1 *N* HCl. The amino acid compositions and N-terminal analyses (Table I) indicate that IIA-1 and IIB-1 correspond to residues 240–248 and 239–248, respectively. Peptide IIA-2 is from the segment containing residues 270–277 and peptide IIB-2 from residues 166–169. In each of these peptides the C-terminal residue is nitrated. The electrophoretic mobility of peptide IIB-2 is consistent with the presence of an *N*-succinyllysyl residue.

Nitrotyrosine was recovered from each of the steps employed as follows: 95% after CNBr, 92% after succinylation and desuccinylation, 98% after chymotrypsin digestion, essentially quantitatively from Bio-Gel P-4 and from the antibody columns, 70% for fraction II from DEAE-Sephadex, and 100% for fraction III from DEAE-Sephadex. The overall recovery of nitrotyrosine through the ion-exchange column was approximately 60%. No attempts were made to calculate specific recoveries from high voltage paper electrophoresis. The actual yields and relative yields of each of the peptides analyzed are listed in Table I. The distribution of nitrotyrosine among the various residues of carboxypeptidase is: Tyr-248, 62%; Tyr-169, 20%; Tyr-277, 12%; and Tyr-240, 7%. No other tyrosyl residues were found to be nitrated. Based on an initial nitrotyrosyl content of 1.2 residues per molecule, this would indicate almost 0.75 nitrotyrosyl residue per molecule at Tyr-248.

Method II. Denaturation and Chymotrypsin Digestion. Method I is based on the scheme of Johansen et al. (1972) for isolating an arsanilazotyrosyl-248 peptide from arsanilazotyrosylcarboxypeptidase A. A simplified procedure was devised in an attempt to increase yields and to facilitate the examination of protein nitration in general. First, nitrocarboxypeptidase (100 mg) containing 0.95 nitrotyrosyl residue per molecule was dissolved in 25 ml of 0.05 *M* Tris–2 *M* NaCl (pH 7.5) and denatured by heating for 2 min in a 90° water bath. More than 98% of the protein precipitates under these conditions. The precipitate was washed with 0.1 *M* NH_4HCO_3, resuspended, and digested with chymotrypsin, 2% (w/w), for 24 hr. After acidification and centrifugation, greater than 90% of the original nitrotyrosyl absorbance was found in the supernatant.

Affinity and Ion-Exchange Chromatography. This solution was applied directly to the affinity column and the nitrotyrosyl peptides were obtained as above. These were further fractionated by ion-exchange chromatography on DEAE-Sephadex A-25 (Figure 5) using the 0.02–0.2 *M* NH_4HCO_3 (pH 8.1) gradient system of Peterson and Chiazze (1962). Final purification of the four yellow fractions was by high voltage paper electrophoresis using the pyridine acetate buffer system already described. Sequence assignments (Table II) were again based on amino acid composition and N-terminal residues. Fraction 1 is only compatible with the sequence of residues 166–169. No nitrotyrosine could be detected by amino acid analysis of fraction 2 which represents less than 7% of the total absorp-

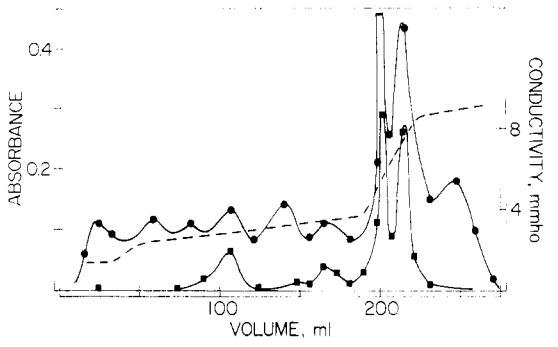

FIGURE 5: Chromatography of nitrotyrosyl peptides on DEAE-Sephadex A-25. The nitrotyrosyl peptide fraction from affinity chromatography of a chymotryptic digest of nitrocarboxypeptidase was applied to a 0.9 × 8 cm column of DEAE-Sephadex A-25 equilibrated with 0.1 *M* NH_4HCO_3 (pH 8.1) and developed according to Peterson and Chiazze (1962). The flow rate was 10 ml/hr and 1.6-ml fractions were collected. Absorbance was measured at 278 (●) and 428 nm (■).

Table II: Amino Acid Composition of Nitrotyrosyl Peptides from a Chymotrypsin Digest of Nitrocarboxypeptidase.

Amino Acid	Fr. 1	(166–169)[a]	Fr. 3	(246–248)[a]	Fr. 4	(247–248)[a]
Lys	1.3	1				
His	1.0	1				
Thr			1.3	1		
Gly	1.4	1				
Ile			1.2	1	1.1	1
Tyr		1		1		1
NO_2-Tyr	1.0		1.0		1.0	
N-Terminus	His		Thr		Ile	
Yield (%)	7		20		21	
Relative yield (%)	13		39		41	

[a] From Bradshaw et al. (1969).

tion eluting from the DEAE-Sephadex column. Fraction 3 represents the sequence Thr-Ile-NO_2Tyr which derives from residues 246–248 while fraction 4 is Ile-NO_2Tyr. There are three possible assignments for an Ile-Tyr sequence in carboxypeptidase but based on the observation that >95% of the nitrotyrosyl label is in the F_1 fragment from CNBr cleavage this particular dipeptide can only represent residues 247–248. The overall yield of nitrotyrosyl peptides from denaturation through the affinity column step is about 85%. This is distributed among fractions 1–4 from the ion-exchange column in the ratio of 13:7:39:41, respectively, in a yield of 60%. Since samples 3 and 4 both originate from that segment of carboxypeptidase containing Tyr-248 and together they account for 80% of the recovered absorption and 86% of the recovered nitrotyrosine, it is clear that Tyr-248 is the residue that reacts most specifically with tetranitromethane.

This same procedure was also employed with a sample of nitrocarboxypeptidase containing 0.5 mol of nitrotyrosine/mol of enzyme. The distribution among fractions 1–4 from the DEAE-Sephadex chromatography was 16:6:38:40. Again, those peptides containing Tyr-248 account for about 80% of the nitrotyrosine label.

Discussion

The two major peptides purified according to method I were from the segment of the enzyme containing Tyr-248, consistent with previous studies demonstrating the unusual susceptibility of this residue to chemical modification (Johansen et al., 1972; Cueni, 1974). Tyr-169 was also nitrated to an appreciable extent (about 20%) while Tyr-240 and Tyr-277 were nitrated only minimally. One of the tyrosyl residues in the N-terminal CNBr fragment (9, 12, or 19) was also nitrated to a small extent (Figure 1). Although the identity of this residue was not examined, previous studies with arsanilazocarboxypeptidase modified in solution (Wilchek et al., 1971) and with iodocarboxypeptidase (F. A. Quiocho, 1967, unpublished results cited in Quiocho and Lipscomb (1971)) indicated partial modification of Tyr-19. Some of the Ile-NO_2-Tyr isolated by method II may, in fact, arise from Tyr-19.

Method II—which omitted CNBr cleavage and succinylation—also gave two major peptides, residues 246–248 and 247–248, respectively. A third, obtained by method I as well, contained residues 166–169. All but one of the nitrotyrosyl peptides isolated contained a C-terminal nitrotyrosyl residue indicating a high degree of chymotrypsin selectivity for NO_2Tyr–X peptide bonds. Nitrocarboxypeptidase examined by method II contained less than 1.0 nitrotyrosyl residue per molecule of protein, perhaps accounting for the failure to recover peptides containing residues 270–277 and 239–243. The overall recovery of nitrotyrosine by both methods was approximately 60% with most of the loss occurring during ion-exchange chromatography.

The bond between Thr-245 and Thr-246 was cleaved by chymotrypsin in method II but not in method I. Incomplete desuccinylation of an *O*-succinylthreonine residue could be one possible explanation. *O*-Succinylation of threonyl residues in proteins rarely occurs under standard modification conditions (Gounaris and Perlmann, 1967), but with large molar excesses of anhydride as in method I, greater acylation could result. *O*-Succinylthreonine deacylates slower than *O*-succinylserine (Gounaris and Perlmann, 1967) with a marked pH-dependence (Johansen, 1969). Hence, Thr-245 or Thr-246 could remain partially acylated after hydroxylamine treatment. When succinylation was omitted (method II), neither of the longer peptides were isolated.

Direct evidence that Tyr-248 behaves differently from all other tyrosyl residues in carboxypeptidase has previously been obtained by Johansen et al. (1972) who found that coupling of the crystalline enzyme with diazotized arsanilic acid specifically occurs at Tyr-248. There is no apparent change in esterase activity as a consequence of this chemical modification and peptidase activity only decreases by about 25–50%. Similar results have also been obtained by Sokolovsky and Vallee (1967) and by Cueni (1974) using diazotized 5-amino-1*H*-tetrazole. This reagent selectively couples with Tyr-248 (L. Cueni and J. F. Riordan, in preparation), but in this instance esterase activity doubles while peptidase activity decreases by only 10%. A detailed kinetic investigation of these modified carboxypeptidases using a variety of substrates (Auld and Holmquist, 1973) has revealed that the changes in activity are even less pronounced than might be inferred from results obtained under the routine assay conditions used for survey studies. Thus, even though it has been considered an essential catalytic residue, Tyr-248 can be coupled with two different diazonium compounds with only minor effects on enzymatic activity.

Both azo coupling reactions are highly specific underscoring the unique nature of this tyrosyl residue. Since other tyrosines in carboxypeptidase accessible to solvent are not modified (Quiocho and Lipscomb, 1971), it would seem that Tyr-248 is hyperreactive, perhaps due to aspects of its

immediate chemical environment. The same high degree of selectivity toward two different diazonium compounds makes it improbable that the reagents themselves determine the specificity of the reaction. This selectivity also pertains to nitration with tetranitromethane.

Nitration affects both the peptidase and the esterase activity of carboxypeptidase. With the conventional peptide substrate, benzyloxycarbonylglycyl-L-phenylalanine, activity is decreased to about 10–20% of the control while with the ester, hippuryl L-phenyllactate, activity increases to 180% under standard assay conditions. Clearly the integrity of Tyr-248 is not essential to catalysis. Any derivative of the enzyme in which this residue is known to be modified retains at least partial activity, though there is always some effect on one or both activities. The changes do not correlate with phenolic hydroxyl group pK and they differ for different substrates. Often there is an effect on substrate binding but changes in k_{cat} have also been noted (Auld and Holmquist, 1973).

In solution, the titration behavior of nitrocarboxypeptidase is typical of nitrophenol ionization, except that its apparent pK is 6.3 rather than 7.0 (Riordan et al., 1967). We have proposed that charge effects in the local environment of the nitrotyrosyl residue could account for this decrease in apparent pK (Riordan and Muszynska, 1974). In contrast to the enzyme in solution, the nitrotyrosyl ionization of nitrocarboxypeptidase in the crystalline state has an apparent pK of 8.2. This marked change in titration behavior, brought about solely by a change in the physical state of the enzyme, has suggested that the conformations of Tyr-248 in crystals and in solution are different (Riordan and Muszynska, 1974). The proximity of Tyr-248 to the zinc ion could readily account for its enhanced reactivity toward tetranitromethane in solution and its low pK after nitration. The positive charge on the cation could promote ionization of the phenolic hydroxyl group and facilitate nitration. Only the ionized form of tyrosine reacts with tetranitromethane (Riordan et al., 1966; Bruice et al., 1968) and the rate of nitration of tyrosyl copolymers as well as the apparent pK of the resultant nitrotyrosyl hydroxyl group both depend on the net charge of the copolymer (Vallee and Riordan, 1968).

On the other hand, Tyr-248 does not interact with zinc when the enzyme is in the crystalline state, yet its modification is still highly specific. The x-ray structural model of carboxypeptidase provides a plausible rationale. Rotating the C_α–C_β bond of Tyr-248 by approximately 120° brings the phenolic hydroxyl group very close to Arg-145, and its positive charge could thus facilitate nitration. Moreover, hydrogen bonding between the resultant nitrophenol and the guanidino group could stabilize the phenolic proton and lead to the increased pK observed for the nitroenzyme crystals. No hindrance to the proposed rotation is obvious.

The structural locations of Tyr-169, Tyr-240, and Tyr-277, the other residues found to be nitrated, albeit partially, support this view. Tyr-169 lies on the enzyme surface with its phenolic hydroxyl group between the ϵ-amino groups of Lys-168 and Lys-177. Tyr-240 is potentially close to the ϵ-amino group of Lys-224, assuming free rotation in the lysyl side chain, and Tyr-277 is immediately adjacent to the guanidino group of Arg-276. Thus, in each case there is a positive charge in the vicinity of the nitratable tyrosine. Except for Tyr-248 no other tyrosyl residue in the enzyme is either nitrated or near a positive charge. It would seem likely that a neighboring cation, be it the zinc ion or Arg-145, is an important factor in the nitration of Tyr-248.

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