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# The Structure and Function of Carboxypeptidase $C_N$

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Carboxypeptidase $C_N$ from *Citrus natsudaidai* HAYATA is an acidic protease ($M_r$ 93000). The enzyme was inactivated by the incorporation of 1.0 mol of $^{32}$P-labeled diisopropylphosphofluoridate, 1.3 mol of $^{203}HgCl_2$, or 2.2 mol of $^{110m}AgNO_3$ per mol of enzyme at pH 5.5. Four or five radioactive peptides were isolated from a partial acid hydrolysate of the $^{32}$P-labeled enzyme. These peptides provided evidence indicating the amino acid sequence Glx–Gly–Asx–Ser–Gly–Gly–Glu–Leu–Val around the reactive serine residue.

The enzyme reacted with 0.4 mol of $^{203}$Hg-labeled *p*-chloromercuribenzoate without appreciable loss of enzymatic activity. On the other hand, 1 mol of $^{203}$Hg-labeled *p*-chloromercuribenzoate was incorporated into the enzyme which had been denatured with 6 M guanidine hydrochloride and 8 M urea at pH 7.0 in the absence of dithiothreitol. Thus, the enzyme has one sulfhydryl group which possesses only poor reactivity.

Photooxidation using methylene blue as a sensitizer caused a loss of enzymatic activity and specific destruction of approximately 1 mol of histidine residue per mol of enzyme. The photooxidized, *p*-bromophenacyl bromide-treated and $HgCl_2$-treated enzyme failed to react with $^{32}$P-labeled diisopropylphosphofluoridate. From these findings, we inferred that one serine, one histidine, and the carboxyl groups are essential for catalytic activity.

**Keywords**——carboxypeptidase; *Citrus natsudaidai*; active site; sequence; photooxidation; chemical modification

Carboxypeptidase $C_N$ [EC 3.4.16.1], a nonspecific glycoprotein exopeptidase from the exocarp of *Citrus natsudaidai* HAYATA, has the ability to liberate most amino acids (including proline) sequentially from the carboxy terminus of a peptide chain.[1–3] This ability, along with the high stability to heat, has made this enzyme especially useful for protein sequencing studies. The enzyme is sensitive to diisopropylphosphofluoridate ($iPr_2PF$), $HgCl_2$, and $AgNO_3$ as well as to modification either with *p*-bromophenacyl bromide or with glycine ethyl ester hydrochloride and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide.[2] It is not affected by ethylenediaminetetraacetic acid (EDTA), 1,10-phenanthroline, *N*-ethylmaleimide, *p*-chloromercuribenzoate (*p*CMB), dithiothreitol, or monoiodoacetic acid at its pH optimum of 5.5.[1,2] These findings suggest that carboxypeptidase $C_N$ is a serine protease requiring carboxyl groups for enzymatic activity.

Similar carboxypeptidases have been purified to homogeneity from higher plants.[4–11] Most of them share the properties of being $iPr_2PF$-sensitive, having broad specificity, and showing activity in the acidic pH region; thus, their active site appears to be unique. The amino acid sequence around the reactive serine of a carboxypeptidase, phaseolaine, from French beans was determined to be Glu–Ser–Val.[9] A reactive serine residue was also identified in carboxypeptidases $C_{Ua}$ and $C_{Ub}$ from the exocarp of *Citrus unshiu* MARC.[12] There is, however, little information available concerning the active site of the plant carboxypeptidases for elucidation of the catalytic mechanism.

The purpose of the present work was to determine the amino acid sequence around the reactive serine residue and to identify the amino acid residues having essential carboxyl groups in carboxypeptidase $C_N$ in order to obtain information on the active site. The mode of inhibition by $HgCl_2$ and $AgNO_3$ was also investigated by the use of labeled compounds. The results indicate that one serine, one histidine, and carboxyl groups are involved in the active site of the enzyme.

## Materials and Methods

Carboxypeptidase $C_N$ from the exocarp of *Citrus natsudaidai* HAYATA was purified to homogeneity according to Kubota *et al.*[1] Carbobenzoxy (Cbz)-Glu–Phe was obtained from the Protein Research Foundation, Osaka. Bovine plasma albumin (Fraction V) was purchased from Armour Pharmaceutical Co., Kankakee, Illinois. $HgCl_2$, *p*CMB, dansyl chloride, and *p*-bromophenacyl bromide were products of Nakarai Chemical Co., Kyoto. $iPr_2PF$ was purchased from Sigma Chemical Co., St. Louis, Missouri. Phenylisothiocyanate, trifluoroacetic acid, and *n*-butyl acetate (all of sequential grade) were obtained from Wako Pure Chemical Industries, Osaka, as were EDTA, Silica gel G, Scintisol, Methylene blue, and 3-phenylpropionic acid. Blue dextran and various grades of Sephadex were obtained from Pharmacia Fine Chemicals, Uppsala. Carboxypeptidase A ($iPr_2PF$-treated) was obtained from P-L Biochemicals Inc., Milwaukee, Wisconsin. [$^{32}$P]$iPr_2PF$ (specific radioactivity, 76.7 $\mu$Ci/$\mu$mol) in propylene glycol, $^{203}HgCl_2$ (specific radioactivity, 263.0 $\mu$Ci/$\mu$mol) in water, and [$^{203}$Hg] *p*CMB (specific radioactivity, 3.0 $\mu$Ci/$\mu$mol), crystalline, were purchased from the Radiochemical Centre, Amersham, England. $^{110m}AgNO_3$ (specific radioactivity, 722.8 $\mu$Ci/$\mu$mol) was obtained from New England Nuclear, Boston, Massachusetts. These radioactive compounds were each diluted with the corresponding unlabeled compounds to a suitable specific radioactivity. The radiochemical purity of these compounds was examined by thin-layer chromatography (TLC). Pronase E, a commercial preparation of *Streptomyces griseus* protease, was obtained from Kaken Chemical Co., Tokyo. All other reagents and organic solvents used were of analytical grade.

**Miscellaneous Methods**

Carboxypeptidase activity was determined with Cbz-Glu–Phe as a substrate according to the procedures described previously.[1] Protein concentration was determined by the method of Lowry *et al.*[13] Paper electrophoresis for purification of peptides was carried out by the method described previously.[12] Peptides were hydrolyzed with 0.2 ml of 6 N HCl in evacuated, sealed tubes at 110 °C for 24 h. Amino acid analysis was performed with 1—10 nmol of the samples on a single column (0.6 cm diameter) of a Hitachi KLA-5 amino acid analyzer according to the manufacturer's instructions. The content of tryptophan was determined on samples hydrolyzed in 6 N $Ba(OH)_2$ at 110 °C for 20 h by the method of Noltmann *et al.*[14] Amino acid sequence analysis was performed by the dansyl-Edman method of Hartley[15] and by using carboxypeptidases.[12] The butyl acetate extracts from Edman degradation containing the phenylthiohydantoin derivatives of amino acids were dried in counting vials and tested for radioactivity. Photooxidation of the enzyme and measurement of the ultraviolet absorption spectrum of the photooxidized enzyme were performed by the methods of Takahashi[16] and Murachi *et al.*[17] Radioactivity of $^{32}$P (Cerenkov radiation) was determined with an Aloka Model ISC-502 liquid scintillation counter, and that of $^{203}$Hg and $^{110m}$Ag with an Aloka Model PS-9 NaI scintillation counter.

**Reaction of Enzyme with [$^{32}$P]$iPr_2PF$ and Partial Acid Hydrolysis of the Products**——Carboxypeptidase $C_N$ (14 mg, 0.14 $\mu$mol) was incubated with [$^{32}$P]$iPr_2PF$ (0.19 ml, 0.5 $\mu$mol) in 2 ml of 0.1 M citrate buffer (pH 5.5) at 35 °C for 120 min. An equal volume of [$^{32}$P]$iPr_2PF$ was then added, and incubation was continued for an additional 60 min under the same conditions. In order to inhibit the enzyme completely, unlabeled $iPr_2PF$ (1 $\mu$mol) was added, and the mixture was incubated for a further 60 min. The final molar ratio of $iPr_2PF$ to enzyme was 7.1. To remove the excess reagent, the mixture was passed through a column (2 × 35 cm) of Sephadex G-25 with distilled water as an eluant. The fractions containing the $^{32}$P-labeled protein were collected and lyophilized. The $^{32}$P-labeled protein was partially hydrolyzed at 110 °C for 40 min in 0.48 ml of 6 N HCl according to the method of Light.[18] The hydrolysate was diluted with 2 ml of distilled water and lyophilized.

**Reaction of Enzyme with $^{203}HgCl_2$, [$^{203}$Hg] *p*CMB, and $^{110m}AgNO_3$**——Aliquots of 0.5—2 ml of the enzyme (8—21 nmol) in 0.1 M citrate buffer (pH 5.5) were added to equal volumes of $^{110m}AgNO_3$ (specific radioactivity, 1 $\mu$Ci/$\mu$mol) and [$^{203}$Hg]*p*CMB (3 $\mu$Ci/$\mu$mol) and to three-quarters volume of $^{203}HgCl_2$ (192 $\mu$Ci/$\mu$mol), and the mixtures were kept at 35 °C for 0.5—2 h. The final concentrations of labeled reagents were $5 \times 10^{-4}$, $1.6 \times 10^{-4}$, and $1.7 \times 10^{-4}$ M $AgNO_3$, $HgCl_2$, and *p*CMB, respectively. In other experiments with *p*CMB, the enzyme was pretreated with 6 M guanidine hydrochloride or 8 M urea at 4 °C and pH 5.5 for 48 h and was dialyzed against 0.1 M citrate buffer (pH 5.5). The pretreated enzyme, which had been completely inactivated, was added to an equal volume of labeled *p*CMB, and the mixture was kept at 35 °C for 2—8 h. To remove the excess reagents, the mixture was passed through a column (0.6 × 15 cm) of Sephadex G-25 with the same buffer as an eluant; furthermore, the effluent was dialyzed against the same buffer containing 1 mM EDTA for 6 d and assayed for enzymatic activity with Cbz-Glu–Phe as a

substrate. Incorporation of $^{110m}AgNO_3$, [$^{203}$Hg] *p*CMB was determined on aliquots of samples with an Aloka Model PS-9 NaI scintillation counter. Control experiments were carried out individually under identical conditions except for omission of either inhibitors for determination of enzymatic activity or enzyme for estimation of radioactivity.

## Results

### Purification of $^{32}$P-Labeled Peptide

Incorporation of $^{32}$P was determined with aliquots of [$^{32}$P]i$Pr_2$PF-treated carboxypeptidase $C_N$ eluted from the Sephadex G-25 column by counting Cerenkov radiation.[19] The enzyme is inactivated by i$Pr_2$PF with the incorporation of 1 atom of phosphorus per mol on the basis of a molecular weight of 93000. The partial acid hydrolysate of $^{32}$P-labeled carboxypeptidase $C_N$ was fractionated by paper electrophoresis at pH 1.8, as shown in Fig. 1. The

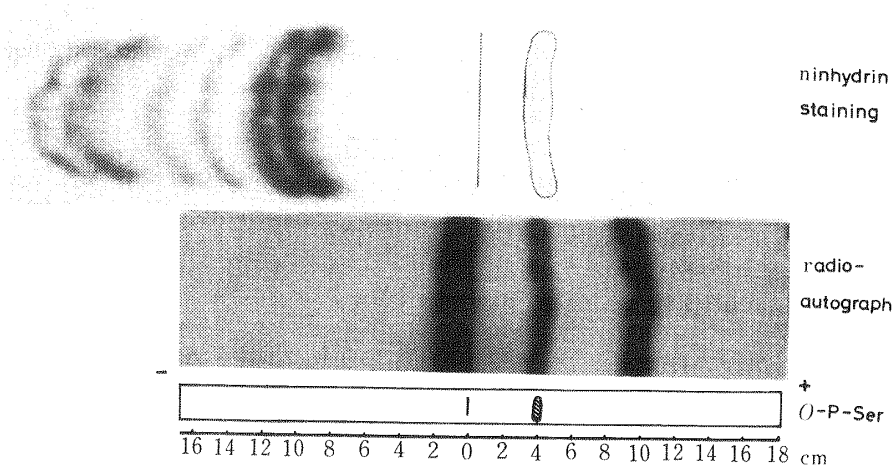


Fig. 1. Paper Electrophoresis of a Partial Hydrolysate of [$^{32}$P]i$Pr_2$PF-Treated Carboxypeptidase $C_N$

The $^{32}$P-labeled enzyme (14 mg, 0.14 $\mu$mol) was hydrolyzed at 110 C for 40 min in 0.48 ml of 6 N HCl. After being diluted with 2 ml of distilled water and then lyophilized, the hydrolysate was dissolved in 0.2 ml of 1 N formic acid (pH 1.8) and applied on a starting line drawn 15 cm from the anode-side edge of the paper (Whatman No. 3MM, 20 × 68 cm). Electrophoresis was carried out in 1 N formic acid (pH 1.8) at 70 V and 4.5 mA per cm for 90 min. After being dried in an oven at 50 C for 60 min, the paper was placed on an X-ray film for 12 h to obtain a radioautograph (middle). Another electrophoretogram obtained under the same electrophoretic conditions was stained with 0.02% ninhydrin in acetone. The electrophoretogram of authentic *O*-P-Ser standard is also shown (bottom).

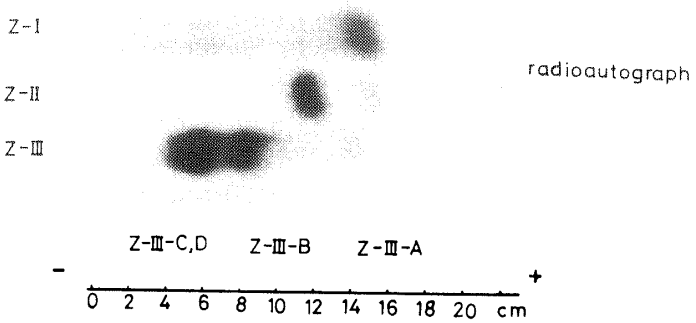


Fig. 2. Paper Electrophoresis of Fractions Z-I, Z-II, and Z-III

Radioactive fractions, Z-I, Z-II, Z-III, were applied on a starting line drawn 20 cm from the anode-side edge of the paper (Whatman No. 3MM, 12 × 68 cm). Electrophoresis was carried out in pyridine–acetic acid–water (1 : 10 : 85, pH 3.6) at 140 V and 4 mA per cm for 120 min. Radioautography of the electrophoretogram was accomplished with an X-ray film by exposure for 24 h.

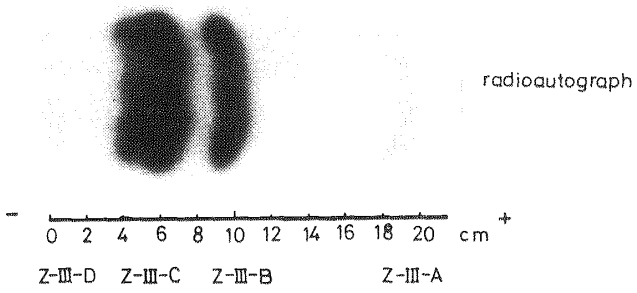


Fig. 3. Paper Electrophoresis for Further Purification of Fraction Z-III

Paper electrophoresis was carried out and the radioautograph prepared under the same conditions as described in the legend to Fig. 2, except that the duration of electrophoresis was increased to 180 min.

TABLE I. Purification and Amino Acid Composition of Radioactive Peptides from a Partial Acid Hydrolysate of [$^{32}$P]$iPr_2PF$-Treated Carboxypeptidase $C_N$

| Purification stage | Total radioactivity (cpm) | Peptide[a] Amount (nmol) | Yield (%) | Amino acid composition |
|---|---|---|---|---|
| Acid hydrolysate | 1183000 | 140[b] | 100 | |
| Paper electrophoresis at pH 1.8[c] | | | | |
| Z-I | 280400 | | | None |
| Z-II | 147900 | 17.5 | 12.5 | Ser 1.0[e] |
| Z-III | 591500 | | | |
| Paper electrophoresis at pH 3.6[d] | | | | |
| Z-III-A | 7100 | | | None |
| Z-III-B | 110000 | 13.0 | 9.3 | Asp 1.0, Ser 1.2 Glu 1.2, Gly 2.2 |
| Z-III-C | 207000 | 24.5 | 17.5 | Asp 1.0, Ser 1.0, Glu 1.7, Gly 2.8, Leu 1.2 |
| Z-III-D | 53200 | 6.3 | 4.5 | Asp 1.0, Ser 1.0, Glu 2.3, Gly 2.8, Leu 1.3, Val 1.3 |

*a*) The yield of peptides was calculated from the radioactivity measurements. *b*) The molecular weight of carboxypeptidase $C_N$ was taken as 93000.[1] *c*) See Fig. 1. *d*) See Fig. 3. *e*) Mol per mol of peptide.

radioautograph revealed three main radioactive fractions (Z-I, Z-II, and Z-III) in a total yield of 86%.

Fractions Z-I (ninhydrin-negative) and Z-II (ninhydrin-positive) migrated 10 and 4 cm, respectively, from the starting line to the anode. The latter was identified as *O*-[$^{32}$P]phosphorylserine (*O*-$^{32}$P-Ser) by comparison with an authentic standard. Fraction Z-III migrated 1 cm from the starting line to the cathode. Each radioactive fraction was pooled and further purified by paper electrophoresis at pH 3.6. As shown in Fig. 2, fraction Z-I gave a single radioactive band which trailed, whereas fraction Z-II exhibited a major radioactive band preceded by a faint band. Fraction Z-III, which was still heterogenous, was again subjected to paper electrophoresis at pH 3.6 for 180 min, yielding four radioactive bands (fractions Z-III-A, Z-III-B, Z-III-C, and Z-III-D) in a total yield of 64% as shown in Fig. 3. The homogeneity of each fraction was established by re-paper electrophoresis performed under the same conditions.

Radioactive sections of the paper were cut out and extracted with 30% acetic acid. The extracts were lyophilized and subjected to amino acid analysis as described under Materials and Methods. The observed values of amino acids were corrected for the blank values from a corresponding section of the paper. Table I summarizes the purification and amino acid composition of the peptides. No amino acid was detected in fraction Z-I or Z-III-A.

TABLE II. Amino Acid Sequence around Reactive Serine Residue at the Active Site of Carboxypeptidase $C_N$

| | 1 Glx | 2 Gly | 3 Asx | 4 Ser[a] | 5 Gly | 6 Gly | 7 Glu | 8 Leu | 9 Val |
|---|---|---|---|---|---|---|---|---|---|
| DNS-Edman degradation[b] | | | | | | | | | |
| Z-III-B | | | → | → | → | → | - - → | | |
| Z-III-C | → | → | → | | | | | | |
| Z-III-D | → | → | → | → | → | - - → | | | |
| Carboxypeptidase $C_N$ digestion | | | | | | | | | |
| Cbz-Z-III-B (35 °C, 10 h) | | | ← - - | ← (0.3) | ← | ← (0.8) | ← (1.0) | | |
| Z-III-C (35 °C, 3 h) | | | | | | | | ← | |
| Cbz-Z-III-C (35 °C, 24 h) | | | | | | | ← - - | ← (0.5) | |
| Cbz-Z-III-D (35 °C, 24 h) | | | | | | | ← | ← (0.3) | ← (0.6) |
| Carboxypeptidase A digestion | | | | | | | | | |
| Cbz-Z-III-C (35 °C, 24 h) | | | | | | | ← - - | ← (0.4) | |
| Cbz-Z-III-D (35 °C, 24 h) | | | | | | | | | ← (0.4) |

*a*) Reactive serine. *b*) Results obtained on dansyl (DNS)-Edman degradation and on carboxypeptidase degradation are shown by the arrows → and ←. Dashed arrows (- - →) designate probable sequences.

## Sequence of $^{32}$P-Labeled Peptides

The amino acid sequence of each peptide is given in Table II. The sequence of peptide Z-III-B was determined to be Asx–Ser–Gly–Gly–(Glx) by the dansyl-Edman technique. Peptides Z-III-C and Z-III-D had partial sequences Glx–Gly–Asp and Glx–Gly–Asx–Ser–Gly–(Gly), respectively, the latter giving a good partial overlap between peptides Z-III-B and Z-III-C.

The amino terminus of each peptide was blocked with carbobenzoxyl chloride,[20] and the blocked peptide was used as a substrate. A 10-h carboxypeptidase $C_N$ digestion of Cbz-Z-III-B released 1.0, 0.8, and 0.3 mol of glutamic acid, glycine, and serine, respectively, per mol of peptide, whereas the unblocked peptide, Z-III-B, was not easily cleaved by the enzyme. The C-terminal amino acid residue of Z-III-C was found to be leucine by carboxypeptidase $C_N$ digestion. From Cbz-Z-III-C, carboxypeptidases $C_N$ and A released 0.5 and 0.4 mol, respectively, of leucine after 24 h of digestion. From Cbz-Z-III-D, carboxypeptidase $C_N$ liberated 0.6 and 0.3 mol of valine and leucine, respectively, and carboxypeptidase A released 0.4 mol of valine after 24 h. The complete sequence of the peptide was concluded to be: Glx–Gly–Asx–Ser–Gly–Gly–Glu–Leu–Val.

## Inactivation of Carboxypeptidase $C_N$ by Photooxidation

Carboxypeptidase $C_N$ was rapidly inactivated by methylene blue-catalyzed photooxidation following first-order kinetics, as shown in Fig. 4. The reaction rate constant was $1.6 \times 10^{-4}\,s^{-1}$. After oxidation for 80 min at pH 5.5 and 22 °C, the activity decreased to less than 5% of that of the control. 3-Phenylpropionic acid partially protected the enzyme from inactivation. The residual activity after 80 min was 20% relative to the control when 0.025 M 3-phenylpropionic acid was present in the reaction mixture. The incorporation of [$^{203}$Hg]*p*CMB did not change before and after photooxidation.

The amino acid analysis of extensively photooxidized carboxypeptidase $C_N$ (12% residual activity) showed no appreciable loss of theoretically susceptible tyrosine, tryptophan,

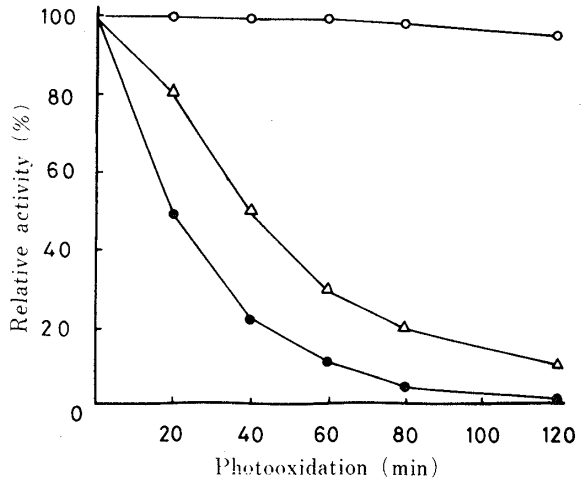


Fig. 4. Time Course of Photooxidative Inactivation of Carboxypeptidase $C_N$

One ml of 0.05% methylene blue aqueous solution was added to 1 ml of carboxypeptidase $C_N$ solution (0.21 mg, 2.25 $\mu$mol) in 0.1 M citrate buffer (pH 5.5) in the presence (●) and absence (△) of 0.025 M 3-phenylpropionic acid. The reaction mixture was irradiated from a distance of 20 cm with a 150 W incandescent lamp at 22 °C. Aliquots of 0.1 ml were withdrawn from the reaction mixture at 20, 40, 60, 80, and 120 min and assayed for enzymatic activity with Cbz-Glu–Phe as a substrate. Control experiments (○) were performed under identical conditions without illumination. Relative activities are expressed as a percentage of the activity of the control.

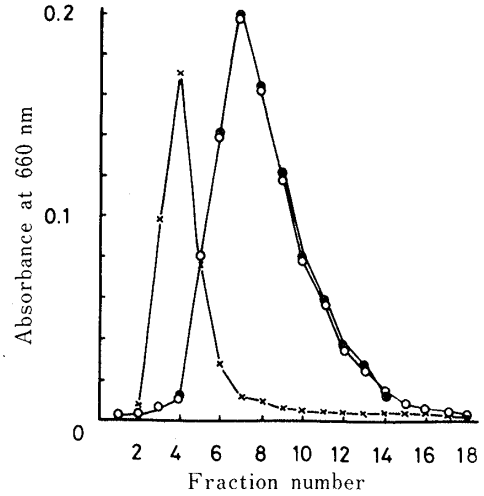


Fig. 5. Gel Filtration of Carboxypeptidase $C_N$ on Sephadex G-100 before and after Photooxidation

The enzyme (0.2 mg/0.2 ml) before (○) and after (●) photooxidation for 80 min in 0.1 M citrate buffer (pH 5.5) was applied to a Sephadex G-100 column (0.6 × 30 cm) equilibrated and eluted with the same buffer at a flow rate of 1.2 ml per h at room temperature. Blue dextran (molecular weight 2000000, 0.4 $\mu$M; shown by ×) was used for measurement of the void volume of the column under the same conditions. Effluent was collected in 0.2 ml fractions. Protein concentration was estimated from the absorbance at 660 nm.[13]

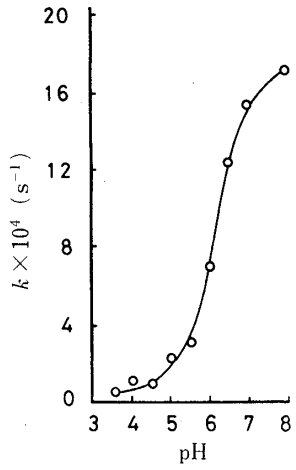


Fig. 6. pH Dependence of the Photoinactivation Reaction

The reaction mixture containing 1.15 $\mu$M carboxypeptidase $C_N$, 16 $\mu$M methylene blue, and 0.1 M citrate buffer (pH 3.5—8.0) in a total volume of 2 ml was irradiated from a distance of 20 cm with a 150 W incandecent lamp at 22 °C for 0—50 min. During this time period, aliquots of 0.1 ml were withdrawn at intervals of 10 min and assayed for enzymatic activity with Cbz-Glu–Phe as a substrate. The rate of inactivation is presented in terms of the first-order rate constants, $k$.

methionine, or cysteine residues. A histidine residue, on the other hand, exhibited a remarkable sensitivity to photooxidation, and approximately 1 mol of histidine residues was destroyed per 1 mol of enzyme.

A portion of the 80 min oxidized sample was applied to a Sephadex G-100 column equilibrated and eluted with 0.1 M citrate buffer (pH 5.5). As Fig. 5 shows, the behavior of the enzyme on Sephadex G-100 was unaffected by photooxidation.

The first-order rate constant of photoinactivation was found to be extremely pH-dependent, as shown in Fig. 6. The inflection point of the curve was at pH 6.3.

**Effect of Chemical Modification on Reactivity**

Carboxypeptidase $C_N$ was inactivated by the incorporation of 1.0 mol of [$^{32}$P]i$Pr_2PF$,

TABLE III. Reaction of [$^{32}$P]$iPr_2PF$ with Carboxypeptidase $C_N$ Inactivated by *p*-Bromophenacyl Bromide, $HgCl_2$, and Photooxidation

| Treatment[a] or reagent | Concentration of reagent (mM) | Relative[b] enzymatic activity (%) | Specific radio-activity (cpm/nmol) | Relative radio-activity (%) |
|---|---|---|---|---|
| *p*-Bromophenacyl bromide | 0 | 100 | 1950 | 100 |
| | 1 | 42 | 741 | 38 |
| $HgCl_2$ | 0 | 100 | 1650 | 100 |
| | 0.1 | 30 | 1100 | 67 |
| | 1 | 2 | 280 | 17 |
| Photooxidation | | 100 | 1800 | 100 |
| | | 55 | 954 | 53 |
| | | 37 | 670 | 37 |
| | | 24 | 449 | 25 |

*a*) The enzyme was modified with *p*-bromophenacyl bromide and $HgCl_2$ according to the method described previously (1, 2) and photooxidized as described under Materials and Methods. Then 0.05 ml of [$^{32}$P]$iPr_2PF$ (60 $\mu$Ci/$\mu$mol/ml) in 0.1 M citrate buffer (pH 5.5) was added to the treated enzyme (28 nmol, 2—8 ml), and the mixture was kept at 35 °C for 3 h. In order to inhibit the enzyme completely, unlabeled $iPr_2PF$ was added to a concentration of 1 mM. The mixture was then dialyzed against 0.1 M citrate buffer (pH 5.5) for 3 d and assayed for radioactivity. Control experiments were carried out under the same conditions except for omission of the treatment or reagent. *b*) Relative enzymatic activities, determined with Cbz-Glu–Phe as a substrate, are expressed as a percentage of the activity of the control.

TABLE IV. Reaction of $^{203}HgCl_2$ with Carboxypeptidase $C_N$ Inactivated by $iPr_2PF$, *p*-Bromophenacyl Bromide and Photooxidation

| Treatment[a] or reagent | Concentration of reagent (mM) | Relative[b] enzymatic activity (%) | Specific radio-activity (cpm/nmol) | Relative radio-activity (%) |
|---|---|---|---|---|
| $iPr_2PF$ | 0 | 100 | 207 | 100 |
| | 1 | 1 | 243 | 117 |
| *p*-Bromophenacyl bromide | 0 | 100 | 318 | 100 |
| | 1 | 45 | 245 | 77 |
| Photooxidation | | 100 | 258 | 100 |
| | | 37 | 420 | 163 |
| | | 8 | 520 | 202 |

*a*) The enzyme was modified with $iPr_2PF$ and *p*-bromophenacyl bromide according to the methods described previously (1, 2) and photooxidized as described under Materials and Methods. Then 0.01 ml of $^{203}HgCl_2$ (193 $\mu$Ci/$\mu$mol/ml) was added to the treated enzyme (12 nmol, 2—8 ml), and the mixture was kept at 35 °C for 30 min. In order to inhibit the enzyme completely, unlabeled $HgCl_2$ was added at a concentration of 1 mM. The mixture was desalted as described under Materials and Methods. Control experiments were carried out under the same conditions except for omission of the treatment or reagent. *b*) Relative enzymatic activities, determined with Cbz-Glu–Phe as a substrate, are expressed as a percentage of the activity of the control.

1.3 mol of $^{203}HgCl_2$, or 2.2 mol of $^{110m}AgNO_3$ per mol of enzyme. The intact enzyme reacted with 0.4 mol of [$^{203}$Hg]*p*CMB, whereas 1.0 mol was incorporated into the enzyme after denaturation with 6 M guanidine and 8 M urea.

Carboxypeptidase $C_N$ inactivated by *p*-bromophenacyl bromide,[21] $HgCl_2$, or photooxidation did not readily react with [$^{32}$P]$iPr_2PF$, as shown in Table III. In every case, the decrease in enzymatic activity was accompanied by a decrease in the incorporation of [$^{32}$P]-$iPr_2PF$. In contrast to this, the enzyme inactivated by $iPr_2PF$ reacted with $^{203}HgCl_2$ regardless of the loss in enzymatic activity, as shown in Table IV. The incorporation of $^{203}HgCl_2$ into the

*p*-bromophenacyl bromide-treated enzyme, however, decreased with decrease in the enzymatic activity, and, conversely, the incorporation of $^{203}HgCl_2$ into $iPr_2PF$-treated enzyme increased with decrease in the enzymatic activity.

Similar tendencies were observed also with $^{110m}AgNO_3$. The $iPr_2PF$-treated enzyme reacted with $^{110m}AgNO_3$. In contrast to the case with $HgCl_2$, however, the incorporation of $^{110m}AgNO_3$ did not increase after photooxidation.

## Discussion

### Amino Acid Sequence of $^{32}P$-Labeled Peptides

Carboxypeptidase $C_N$ is inactivated by $iPr_2PF$ with incorporation of 1 atom of $^{32}P$ per mol on the basis of a molecular weight of 93000. One radioactive amino acid and three radioactive peptides were obtained from the partial acid hydrolysate of the $^{32}P$-labeled protein by paper electrophoresis. The radioactive amino acid residue was identified as serine. Thus, the $iPr_2PF$ had reacted with a serine in carboxypeptidase $C_N$. These radioactive peptides provide evidence of the amino acid sequence Glx–Gly–Asx–Ser–Gly–Gly–Glu–Leu–Val around the reactive serine residue of the enzyme, and this sequence is identical to that of carboxypeptidase $C_{Ua}$[12] from mandarin orange. It remains to be determined, however, whether the aspartic and glutamic acids are present in the enzyme as the free acids or as their amide derivatives.

The amino acid sequence around the reactive serine residue of carboxypeptidase $C_N$ was similar in part to those of trypsin (Gln–Gly–Asp–Ser–Gly–Gly–Pro–Val–Val)[22] and chymotrypsin (Met–Gly–Asp–Ser–Gly–Gly–Pro–Leu–Val),[23] but differed from those of phaseolain (Glu–Ser–Val)[9] and carboxypeptidase Y (Ala–Gly–Glu–Ser–Tyr–Ala–His–Gly–Tyr), which was isolated from baker's yeast by Hayashi *et al.*[24]

### Photooxidation

Histidine, cysteine, methionine, tyrosine, and tryptophan are known to be photooxidized in the presence of methylene blue or rose bengal as a sensitizer.[25–27] Among these photosensitive amino acid residues, only histidine shows a marked pH dependence in its destruction, and the pH dependence in the pH region of 4.0 to 8.0 is characteristic of imidazole photooxidation.[28] The oxidation of tryptophan causes a marked decrease in absorption at 280 nm, and a definite change in the range of 260—280 nm is observed with tyrosine after photooxidation.[29]

Photooxidation of aspartate aminotransferase in the presence of methylene blue led to a first-order loss of enzymatic activity with specific destruction of a histidine residue, though other photosensitive amino acid residues were also appreciably affected.[30] We have demonstrated the photooxidation of active carboxypeptidase $C_N$ with the destruction of about 1 mol of histidine residue per mol of enzyme according to first-order kinetics, and we found a pH dependence similar to that of aspartate aminotransferase. The intact and photooxidized carboxypeptidase $C_N$ showed identical ultraviolet spectra and gel filtration behavior on a column of Sephadex G-100. As judged from these findings, neither significant modification of other photosensitive amino acids nor major conformational changes of the enzyme molecule seemed to have taken place. Thus, one histidine residue is essential for enzymatic activity. It is probable that 3-phenylpropionic acid interacts with the active center to protect the histidine residue from photooxidative destruction.

### Characteristics of the Active Site

Mechanisms of proteolytic cleavage of protein were proposed by Blow *et al.*[31] and Kraut,[32] but these mechanisms are not yet well-defined.

Louis *et al.*[33] showed from the results of a crystallographic study that two carboxyl groups (Asp-102 and Asp-194), His-57, and Ser-195 were involved in the catalytic mechanism

of chymotrypsin. Titani *et al.*[34] also demonstrated that bovine trypsin contained three active carboxyl groups (Asp-102, Asp-194, and Asp-189) in the active site. In the case of carboxypeptidase $C_N$, one serine and one histidine residues, and carboxyl groups are essential for its enzymatic activity. The enzyme inactivated by modification of carboxyl groups with *p*-bromophenacyl bromide and by the specific destruction of an imidazole group by photooxidation is not liable to react with [$^{32}$P]i$Pr_2$PF. These findings indicate that a mechanism involving a charge relay system similar to that described by Blow *et al.*[31] for chymotrypsin is working in the carboxypeptidase $C_N$. His system, however, is not complete owing to the protonation of the imidazole group of the histidine residue in the acid region. Thus, we propose that some carboxylates are essential for preventing protonation at the position of the histidine residue leading to the formation of the charge relay system among another carboxylate group of an aspartic acid, the imidazole group of histidine, and the hydroxyl group of a serine residue Hayashi *et al.*[35] reported that one serine and one histidine residues, which probably formed a charge relay system, were involved in the catalytic site of carboxypeptidase Y from baker's yeast.

*p*CMB reacts with a sulfhydryl group.[36] When carboxypeptidase $C_N$ was reacted with labeled *p*CMB, 0.4 mol of labeled *p*CMB was incorporated into 1 mol of enzyme with no loss of enzymatic activity. The enzyme denatured with 6 M guanidine and 8 M urea bound 1.0 and 1.3 mol of labeled *p*CMB per mol of enzyme, respectively. The sulfhydryl group was not oxidized during photooxidation. These results suggest that the sulfhydryl group may be buried in an inner part of the molecule, resulting in poor reactivity of this group.

The enzyme reacted with 1.3 mol of labeled $HgCl_2$ or 2.2 mol of labeled $AgNO_3$ per mol of enzyme with complete loss of activity. $HgCl_2$ is known to react with a sulfhydryl, histidyl, or carboxyl group. Chambers *et al.*[37] also demonstrated in the crystallographical study that $AgNO_3$ was bound between the $\beta$-carboxylate group of Asp-102 and the imidazole group of His-57 in the charge relay system of trypsin. Carboxypeptidase $C_N$ inactivated by $HgCl_2$ and $AgNO_3$ could not be reactivated by dialysis or by the addition of chelating agents. These metal ions might, therefore, be bound tightly in a specific site of the enzyme. In addition, the enzyme treated with *p*-bromophenacyl bromide failed to react with $^{203}HgCl_2$ and $^{110m}AgNO_3$, whereas the $iPr_2PF$-treated enzyme reacted with these labeled compounds. We thus inferred that Hg and Ag ions were bound between the $\beta$-carboxylate group and the imidazole group in the charge relay system of carboxypeptidase $C_N$.

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