[Chem. Pharm. Bull. 31(4)1315—1319(1983)]

The Active Site of Carboxypeptidase C_U. II. Photooxidation of Carboxypeptidases C_{Ua} and C_{Ub}

YUKIHO KUBOTA,[a] TAKAYUKI FUNAKOSHI,*,[a] RYOICHI YOKOYAMA,[b] and SHOZO SHOJI[a]

Faculty of Pharmaceutical Sciences, Kumamoto University,[a] 5-1, Oe-Honcho, Kumamoto 863, Japan and Toyo Jozo Co., Ltd.,[b] 5-13, Shibaura 4-chome, Minato-ku, Tokyo 108, Japan

(Received October 4, 1982)

Carboxypeptidases C_{Ua} and C_{Ub} were both inactivated by photooxidation in the presence of methylene blue at pH 5.5 and 8°C. The inactivation was partially prevented by a competitive inhibitor, 3-phenylpropionic acid, of the enzymes. It seemed unlikely that the enzymes underwent a change in molecular size during the photooxidation on the basis of their behavior in electrophoresis and gel filtration. When the photooxidation was carried out at various pH value ranging from 4.5 to 7.5, the rate of inactivation was found to be pH-dependent and the pH profiles conformed to theoretical titration curves with an apparent pK_a value of 6.5, suggesting that an imidazole group of a histidine residue is essential for the enzymatic activity. The photooxidized enzymes had significantly decreased histidine contents, whereas the contents of other amino acids remained essentially unchanged. It was shown that one histidine residue is involved in each active site of the enzymes.

Keywords——carboxypeptidase; *Citrus unshiu*; active site; essential histidine residue; photooxidation

Carboxypeptidases C_{Ua} and C_{Ub} [EC 3.4.16.1], both isolated from the exocarp of mandarin orange (*Citrus unshiu* MARC.), are non-specific carboxyl-terminal exopeptidases having molecular weights of 96000 and 112000, respectively.[1a] Each of the enzymes has one reactive serine residue in the active site and an identical sequence of eight amino acid residues, Glu–Gly–Asp–Ser–Gly–Gly–Glu–Leu, around this serine.[1c] Modification studies showed that, besides the serine residue, aspartic (or glutamic) acid, arginine, and histidine residues seemed to be involved in the active sites.[1b]

The photooxidation of proteins in the presence of methylene blue is known to cause a rapid modification of histidine and tryptophan residues and a slower modification of tyrosine, methionine, and cysteine residues.[2] Photooxidation studies on enzymes have been reported in detail for ribonucleases T_1[3] and U_1,[4] stem bromelain,[5] papain,[6] and carboxypeptidase A_γ.[7] No attempt, however, has so far been made to photooxidize carboxypeptidase C type enzymes.

The present paper describes the effect of photooxidation in the presence of methylene blue on carboxypeptidases C_{Ua} and C_{Ub} and the identification of the oxidized amino acids involved in the active sites of both enzymes. Recently, rose bengal has been used as a more effective sensitizer for photooxidation than methylene blue. However, we preferred to use the latter in the present experiments, because the photooxidation of the enzymes had to be performed under mild conditions to modify their active sites.

Experimental

Enzymes——Carboxypeptidases C_{Ua} and C_{Ub} were purified from the exocarp of *Citrus unshiu* MARC. by the method described previously.[1a] By this purification method, each enzyme was obtained in the form of a solution in 0.1 M citrate buffer, pH 5.5, and was used as such or after dilution with the same buffer to a desired concentration. This buffer was used throughout the present work, unless otherwise specified. When it was necessary to replace the buffer, solutions of the enzymes were dialyzed against the required buffer.

Materials——Benzyloxycarbonyl-L-glutamyl-L-phenylalanine (Z–Glu–Phe) was obtained from the Peptide Research Foundation, Osaka; methylene blue from Katayama Chemical Industries Co., Osaka;

3-phenylpropionic acid (3-PPA) from Tokyo Kasei Kogyo., Tokyo; bovine serum albumin from Armour Pharmaceutical Co., Kankakee, Illinois. Other reagents and organic solvents used were of analytical grade.

Enzyme Assay——Carboxypeptidase activity was determined as described in a previous paper with Z-Glu-Phe as a substrate.[8] The protein concentration of enzyme solution was determined by the Folin-Lowry method at 660 nm.[9] Bovine serum albumin was used as a protein standard.

Photooxidation——Photooxidation of carboxypeptidases C_{Ua} and C_{Ub} was performed according to the method of Takahashi *et al.*[3a] as follows. To 1 ml of 0.006 to 0.073% solution of an enzyme in 0.1 M citrate buffer, pH 5.5, 1 ml of 0.0025 or 0.05% methylene blue was added, and the mixture was kept below 8°C in a circulating water bath with constant stirring. ihe reaction mixture was irradiated from a distance of 20 cm with a 150 W incandescent lamp. Aliquots of 0.1 ml were withdrawn at appropriate time intervals for the assay of enzymatic activity. Control experiments were performed under the same conditions without irradiation or methylene blue.

Amino Acid Analysis——The reaction mixture after photooxidation was dialyzed against distilled water for 4 d at 4°C to remove the reagents and then lyophilized. The protein sample thus obtained was hydrolyzed in an evacuated sealed tube with 0.5 ml of 6 N HCl for 24 h at 110°C. After removal of the HCl, amino acids were determined with a Hitachi KLA-5 amino acid analyzer according to the procedure of Spackman *et al.*[10]

Ultraviolet Absorption Spectrum——The reaction mixtures before and after photooxidation in the presence of 0.00125% methylene blue were dialyzed against distilled water for 3 d to remove the dye. The absorption spectra of the resulting colorless protein solutions were compared. Ionization of phenolic hydroxyl groups in the sample protein was determined by the method of Tachibana and Murachi[11] with a Hitachi model EPS-3T spectrophotometer.

Results

Inactivation of Carboxypeptidases C_{Ua} and C_{Ub} by Photooxidation

Figure 1 shows time courses of photooxidative inactivation of carboxypeptidases C_{Ua} and C_{Ub}. Both enzymes were progressively inactivated by photooxidation in the presence of methylene blue. After 120 min of photooxidation, the activities of carboxypeptidases C_{Ua} and C_{Ub} had decreased to 3 and 10% of those of the controls, respectively, whereas no inactivation took place in the control experiments without methylene blue.

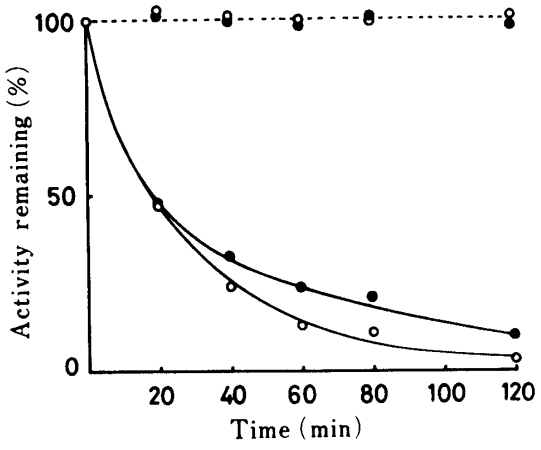

Fig. 1. Time Courses of Photooxidative Inactivation of Carboxypeptidases C_{Ua} and C_{Ub}

Carboxypeptidases C_{Ua} (0.004 %) and C_{Ub} (0.003%) in 0.05 M citrate buffer, pH 5.5, were each photooxidized in the presence of 0.025% methylene blue at 8°C. Control experiments were performed under the same conditions without methylene blue. ○, carboxypeptidase C_{Ua}; ●, carboxypeptidase C_{Ub};——, with methylene blue; ----, without methylene blue.

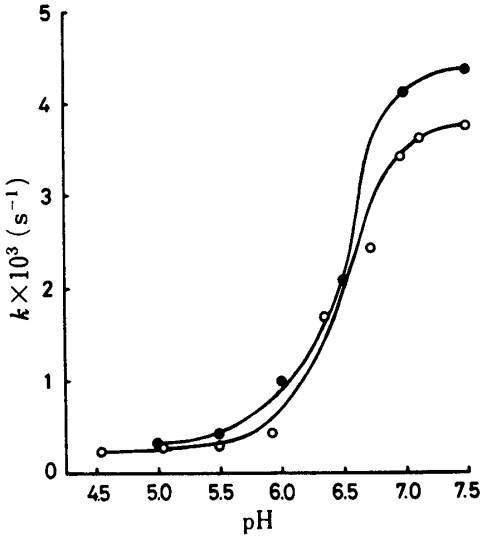

Fig. 2. pH Profiles for Photooxidation of Carboxypeptidases C_{Ua} and C_{Ub}

Photooxidation was performed with 0.006 % protein and 0.025 % methylene blue in 0.05 M citrate buffer for 0—50 min at 8°C. The rate of inactivation is presented in terms of the first-order rate constant, k. ○, carboxypeptidase C_{Ua}; ●, carboxypeptidase C_{Ub}.

Alteration of Molecular Size and Integrity of Carboxypeptidases C_{Ua} and C_{Ub} by Photooxidation

The elution profile for gel filtration of photooxidized carboxypeptidase C_{Ua} on Sephadex G-100 showed a symmetrical peak, as in the case of the native enzyme, and no subpeak was observed in either case. A similar result was obtained with carboxypeptidase C_{Ub}.

Cellulose acetate paper electrophoreses of carboxypeptidases C_{Ua} and C_{Ub} before and after photooxidation were performed by the method described in a previous paper.[1a] Each enzyme showed a single band, and no difference was found in the migration distance before and after photooxidation.

Ultraviolet Absorption Spectra before and after Photooxidation

Carboxypeptidases C_{Ua} and C_{Ub} each showed a typical absorption maximum near 280 nm as described previously.[1b] The ultraviolet absorption spectra on either side of 280 nm did not change after oxidation for 120 min at pH 5.5 and 8°C, indicating that no oxidative destruction of tryptophan or tyrosine residues had taken place.

pH Dependence of Photoinactivation Reaction

The inactivation was found to be a first-order reaction by measurement of the rate constant, k, the values of which, determined in the pH range from 4.5 to 7.5, are given in Fig. 2. The inactivation reaction is clearly pH-dependent. The solid lines in Fig. 2 represent the theoretical titration curves which best fit the experimental data. This indicates that a group having a pK_a value of 6.5 participates in the inactivation of both carboxypeptidases C_{Ua} and C_{Ub}.

Decrease in Histidine Content by Photooxidation

During the photooxidation of carboxypeptidases C_{Ua} and C_{Ub} for 0—30 min at 8°C and pH 5.5, aliquots of the reaction mixtures were withdrawn at appropriate time intervals for amino acid analysis. The only significant changes were in the histidine contents of both enzymes after and photooxidation; the contents of other amino acids were essentially unchanged. Figure 3 shows the relationship between the remaining activity and the loss of histidine of carboxypeptidases C_{Ua} and C_{Ub}, which originally contain 25 and 26 histidine residues,

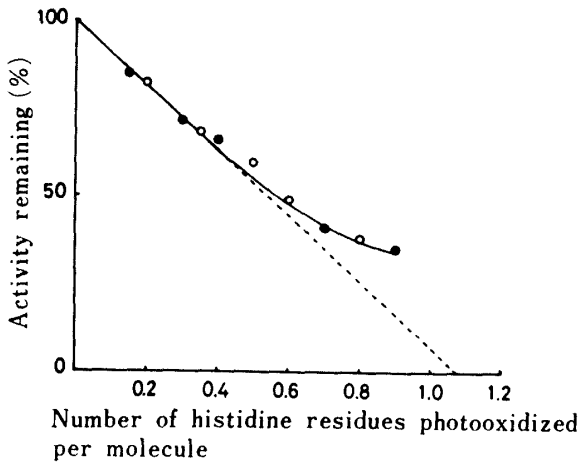

Fig. 3. Relationship between Remaining Activity and Loss of Histidine Content of Carboxypeptidases C_{Ua} and C_{Ub}

The broken line shows the theoretical curve, assuming a linear correlation between the inactivation and the loss of histidine. ○, carboxypeptidase C_{Ua}; ●, carboxypeptidase C_{Ub}.

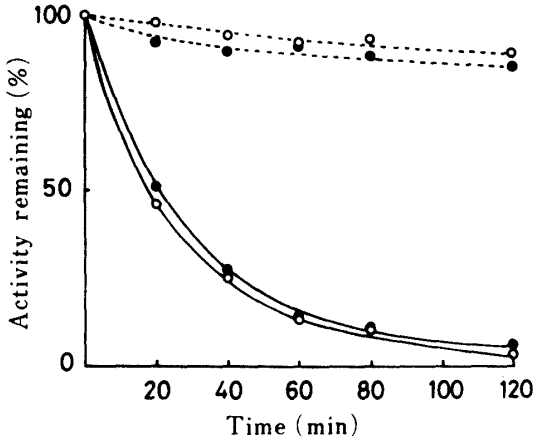

Fig. 4. Effect of 3-PPA on Photooxidation of Carboxypeptidases C_{Ua} and C_{Ub}

Photooxidation was performed with 0.003 % protein and 0.025 % methylene blue in 0.05 M citrate buffer, pH 5.5, at 8°C in the presence or absence of 3-PPA. At the specified intervals, an aliquot of reaction mixture was dialyzed against 0.1 M citrate buffer, pH 5.5, for 48 h at 4°C to remove the reagents and assayed for carboxypeptidase activity. ○, carboxypeptidase C_{Ua}; ●, carboxypeptidase C_{Ub}; ——, without 3-PPA; ----, with 50 mM 3-PPA.

respectively. As the broken line in Fig. 3 shows, the elimination of approximately one histidine residue by photooxidation leads to the complete loss of enzymatic activity. This suggests that one histidine residue participates in the active site of each enzyme.

Effect of Substrate Analog on Photooxidation of Carboxypeptidases C_{Ua} and C_{Ub}

Figure 4 shows that 3-PPA, a substrate analog, has a protective effect on the inactivation of carboxypeptidases C_{Ua} and C_{Ub} by photooxidation. When the enzymes were photooxidized for 120 min at pH 5.5 and 8°C, they lost most of their original activities. However, when 50 mM 3-PPA was present in the reaction mixtures, the enzymes retained 85 to 90% of their original activities.

Discussion

In previous papers,[1] we have reported the chemical and enzymatic properties of carboxypeptidases C_{Ua} and C_{Ub} isolated from the exocarp of mandarin orange. Both enzymes are serine proteases having the ability to liberate most amino acids, including proline, from the C-termini of peptide chains. The enzymes have one reactive serine residue in the active site, and they have identical sequences of seven amino acid residues around the serine in spite of some differences in their chemical and enzymatic properties. This sequence is similar to those of trypsin,[12] chymotrypsin,[13] elastase,[14] and thrombin.[15] Besides the serine residue, a histidine residue and an aspartic acid residue are involved in the active site of each of these serine proteases to form the charge relay system. It was therefore expected that carboxypeptidases C_{Ua} and C_{Ub} would also contain these two amino acid residues in their active sites.

Since Weil *et al.* performed the photooxidation of amino acids[2a] and lysozyme[2b] sensitized by methylene blue, a number of attempts have been made to photooxidize enzymes in the presence of a sensitizer. As regards the photooxidation of carboxypeptidases, ultraviolet irradiation of carboxypeptidase A in the presence of Brij-35 was first reported by Fujioka and Imahori,[16] who suggested a possible relationship between the inactivation of the enzyme and loss of the tryptophan and tyrosine residues. Freude[7] reported that the photooxidation of carboxypeptidase A_γ sensitized by rose bengal or methylene blue caused a pH-dependent progressive inactivation, giving a sigmoidal pH profile which was almost identical with that for photooxidation of histidine. Amino acid analysis of photooxidized carboxypeptidase A_γ indicated that histidine was the only amino acid that was destroyed to any appreciable degree. Tryptophan, tyrosine, cysteine, and methionine residues remained practically unchanged when compared with control hydrolysates.

No attempt, however, has so far been made to photooxidize carboxypeptidase C type enzymes. In the present study, carboxypeptidases C_{Ua} and C_{Ub} were progressively inactivated by methylene blue-catalyzed photooxidation under mild conditions, at pH 5.5 and 8°C. This inactivation pattern was similar to those for the photooxidation of papain,[6] stem bromelain,[5] and carboxypeptidase A_γ.[7] Electrophoresis and gel filtration of carboxypeptidases C_{Ua} and C_{Ub} before and after photooxidation showed that neither significant change in molecular size nor cleavage of peptide bonds occurred during the photooxidation.

Spectral studies of carboxypeptidases C_{Ua} and C_{Ub} before and after photooxidation indicated that tryptophan residues of the enzymes were not modified and that tyrosine residues of the oxidized enzymes could be ionized in the same way as in the unoxidized control.

The effect of pH on the photooxidative inactivation of carboxypeptidases C_{Ua} and C_{Ub} indicated that a group having an apparent pK_a value of 6.5 was involved in the inactivation. This pK_a value is close to that for the photooxidation of histidine, which was shown to be pH-dependent and to give a sigmoidal curve having an inflection near pH 7.0.[17] Tryptophan, tyrosine, or methionine, which is also susceptible to photooxidation, does not show this pH dependence.[17] It is, therefore, not unreasonable to conclude that a histidine residue or

residues are involved in the active sites of carboxypeptidases C_{Ua} and C_{Ub}. The pK_a value of 6.5 is also close to those of stem bromelain,[5] papain,[6] ribonuclease U_1,[4] and carboxypeptidase A_γ[7] which have an essential histidine residue or residues.

Amino acid analysis of carboxypeptidases C_{Ua} and C_{Ub} before and after photooxidation indicated that no amino acid was photooxidized to any appreciable degree except for histidine. There was a correlation between the loss of enzymatic activity and the number of photooxidized histidine residues. For instance, a 50%-inactivated enzyme preparation had lost about 0.6 residue of histidine. The degree of inactivation was lower than the degree of destruction of histidine (Fig. 3). This may be due to the partial destruction of nonspecific histidine residues. Like carboxypeptidases C_{Ua} and C_{Ub}, insulin,[18] enolase,[17] ribonuclease T_1,[3c] and carboxypeptidase A_γ[7] predominantly lost an essential histidine residue or residues located at their active sites during photooxidation.

Furthermore, the fact that 3-PPA, a substrate analog and a competitive inhibitor of carboxypeptidases C_{Ua} and C_{Ub}, protected the enzymes from photooxidative inactivation seems to indicate that the photooxidized histidine residue is located at the active site. Similar protective effects of substrate analogs were observed with guanosine 2′(3′)-phosphate and guanosine 2′-phosphate in the photooxidation of ribonucleases T_1[3b,c] and U_1,[4] respectively. These findings suggest that the protection is primarily due to the binding of the analog to the active site of each enzyme. In the present study, the elimination of approximately one histidine residue resulted in almost complete inactivation of carboxypeptidases C_{Ua} and C_{Ub}. It can be concluded from these results that both enzymes have one essential histidine residue in the active site in addition to one reactive serine residue.[1c]

Acknowledgement This work was supported in part by a grant from the Ministry of Education, Science and Culture of Japan.

References

1) *a*) Y. Kubota, T. Funakoshi, O. Shimooki, and S. Shoji, *Seikagaku*, **47**, 1115 (1975); *b*) Y. Kubota, T. Funakoshi, S. Shoji, M. Moriyama, and H. Ueki, *Chem. Pharm. Bull.*, **28**, 3479 (1980); *c*) T. Funakoshi, S. Shoji, R. Yokoyama, H. Ueki, and Y. Kubota, *Chem. Pharm. Bull.*, **31**, 198 (1983).
2) *a*) L. Weil and A.R. Buchert, *Arch. Biochem. Biophys.*, **34**, 1 (1951); *b*) L. Weil, A.R. Buchert, and J. Maher, *Arch. Biochem. Biophys.*, **40**, 245 (1952); *c*) L. Weil, S. James, and A.R. Buchert, *Arch. Biochem. Biophys.*, **46**, 266 (1953).
3) *a*) S. Yamagata, K. Takahashi, and F. Egami, *J. Biochem.*, **52**, 261 (1962); *b*) K. Takahashi, *J. Biochem.*, **67**, 833 (1970); *c*) M. Irie, *J. Biochem.* (Tokyo), **68**, 69 (1970).
4) J. Hashimoto, K. Takahashi, and T. Uchida, *J. Biochem.* (Tokyo), **73**, 13 (1973).
5) T. Murachi, T. Tsudzuki, and K. Okumura, *Biochemistry*, **14**, 249 (1975).
6) K. Okumura and T. Murachi, *J. Biochem.* (Tokyo), **77**, 913 (1975).
7) K.A. Freude, *Biochim. Biophys. Acta*, **167**, 485 (1968).
8) Y. Kubota, S. Shoji, T. Funakoshi, and H. Ueki, *J. Biochem.* (Tokyo), **74**, 757 (1973).
9) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
10) D.H. Spackman, W.H. Stein, and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).
11) A. Tachibana and T. Murachi, *Biochemistry*, **5**, 2756 (1966).
12) B.S. Hartley, *Philos. Trans. R. Soc. Lond. Ser. B.*, **257**, 77 (1970).
13) J.R. Brown and B.S. Hartley, *Biochem. J.*, **101**, 214 (1966)
14) P.M. Shotton and B.S. Hartley, *Nature* (London), **225**, 802 (1970).
15) S. Magnusson, *Thromb. Diath. Haemorrh., Suppl.* **38**, 97 (1970).
16) H. Fujioka and K. Imahori, *J. Biochem.* (Tokyo), **53**, 341 (1963).
17) E.W. Westhead, *Biochemistry*, **4**, 2139 (1965).
18) L. Weil, T.S. Seibles and T.T. Herskovits, *Arch. Biochem. Biophys.*, **111**, 308 (1965).