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Studies on the Active Site of Papain. VIII.[1] Photooxidation of Tryptophan Residues[2]

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1) Only a tryptophan residue was exclusively modified by photooxidation at pH 4.0 with the loss of enzyme activity.

2) This residue is considered to be identical with the first NBS-oxidizable residue.

3) This residue is different from the residues to be photooxidized at pH 8.0.

4) A tryptophan residue to be photooxidized at pH 4.0 and to be first NBS-oxidized is considered to exist in or nearby the active site of papain. These findings agree with X-ray model of papain.

5) Two tryptophan residues other than the essential residue were modified by photooxidation at pH 8.0 and by NBS oxidation at pH 3.0. These two residues may be exposed in native papain.

6) Other two tryptophan residues were not affected by photooxidation at pH 4.0 and pH 8.0 and by NBS oxidation. It is considered that these two residues are buried in native papain.

7) Based on the interpretation described above, the state of tryptophan residues of papain is illustrated schematically.

Papain [3.4.22.2] possesses a single reactive SH group, a histidine residue and a tryptophan residue, which are considered to play an essential role in the enzyme action. Numerous studies[4] have been carried out on the SH group at the active site of papain. The identification of the histidine residue and the tryptophan residue involved in the active site of papain has been performed by photooxidation[5] and N-bromosuccinimide (NBS) oxidation,[1,6] respectively.

The results of the photooxidation of papain at pH 8.0 in the presence of methylene blue[5] indicated that the loss of histidine causes the inactivation, while the loss of tryptophan does not participate in the inactivation. However, NBS oxidation of papain[1,6] indicated that the loss of tryptophan causes the inactivation. Generally, the rate of photooxidation of histidine is pH dependent,[7] and at the acidic pH, where the imidazole base is protonated, the reaction proceeds only at a very low rate.[8] The rate of photooxidation of indole is not pH dependent.[8] The present research has been planned to clarify the importance of tryptophan residues by means of photooxidation at acidic pH.

Experimental

Enzymes——Crystalline papain was prepared by the procedure of Kimmel and Smith.[9] Mercuripapain was purchased from Sigma Chemical Co., St. Louis.

1) Part VII: M. Sakane, H. Kanazawa and A. Ohara, *Chem. Pharm. Bull.* (Tokyo), **24**, 22 (1976).
2) A part of this research was presented at the 25th Meeting of Kinki Branch, Pharmaceutical Society of Japan, Kobe, Nov., 1975.
3) Location: 5 *Nakauchicho, Misasagi, Yamashina, Higashiyama, Kyoto 607, Japan.*
4) *a*) E.L. Smith and J.R. Kimmel, "The Enzymes," (2 rd ed.) Vol. 4, 1960, pp. 133—172; *b*) A.N. Glazer and E.L. Smith, "The Enzymes," (3 rd ed.) Vol. 3, 1971, pp. 501—546.
5) A. Ohara, S. Fujimoto, H. Kanazawa and T. Nakagawa, *Chem. Pharm. Bull.* (Tokyo), **23**, 967 (1975).
6) M. Sakane, H. Kanazawa and A. Ohara, *Chem. Pharm. Bull.* (Tokyo), **23**, 1741 (1975).
7) E.W. Westhead, *Biochemistry*, **4**, 2139 (1965).
8) L. Weil, *Arch. Biochem. Biophys.*, **110**, 57 (1965).
9) J.R. Kimmel and E.L. Smith, *Biochemical Preparations*, **6**, 61 (1957).

Materials——α-N-Benzoyl-L-arginine amide (BAA) was prepared by the procedure of Kimmel and Smith.[9] NBS, methylene blue, *p*-chloromercuribenzoate (PCMB) and *p*-dimethylaminobenzaldehyde were purchased from Nakarai Chemicals Co. Ltd., Kyoto.

Enzyme Assay——The assay procedure described in the previous paper[10] was employed.

NBS Oxidation——NBS oxidation described by Spande, *et al.*[11] was employed with slight modification.

Photooxidation——Photooxidation was performed at pH 4.0 (0.05 M acetate buffer) by the same procedure as used at pH 8.0 (0.05 M phosphate buffer) in the previous paper.[5]

Amino Acid Analyses——Amino acid analyses were performed by the method of Moore, *et al.*[12] Tryptophan contents were determined by the method of Spies and Chambers.[13]

SH Contents——SH contents were assayed according to the methods of Boyer with PCMB.[14]

Results

Papain was inactivated by methylene blue-sensitized photooxidation at pH 4.0. The inactivation of papain at pH 4.0 was quite similar to that at pH 8.0.[5] Mercuripapain was also inactivated by photooxidation at pH 4.0. However, practically no inactivation of mercuripapain took place at pH 8.0.[5] This fact indicates that the photooxidation at pH 4.0 may be different from that at pH 8.0.

At pH 8.0, one histidine residue per molecule of papain was lost when complete inactivation took place,[5] as shown in Table I. However, amino acid analyses of the papain and the

Table I. Amino Acid Composition of Photooxidized Papain at pH 8.0

Amino acid[a]	Papain		Hg-Papain	
	Intact	Illuminated	Intact	Illuminated
Trp.[b]	5.0	3.5	5.0	3.0
Tyr.	19.0	18.0	19.5	18.3
His.	1.8	1.0	1.9	1.9
-SH[c]	1.0	—	—	—
Met.	0	0	0	0
Activity	100%	0%	100%	100%

a) All amino acids which are present in papain, were examined, but the table reports for those which are known to be susceptible to photooxidation. No change was found in the content of the other amino acids. The values in the table denote number of residues per protein molecule, assuming the number of leucine residue to be 11.0 and the number of arginine residue to be 12.0. No correction was made for decomposition during acid hydrolysis.
b) determined by the method of Spies and Chambers[14]
c) determined by the method of Boyer[13]

Table II. Amino Acid Composition of Photooxidized Papain at pH 4.0

Amino acid	Papain		Hg-Papain	
	Intact	Illuminated	Intact	Illuminated
Trp.	5.0	4.0	5.0	4.0
Tyr.	19.0	19.9	19.5	18.3
His.	1.8	1.9	1.9	1.9
-SH	1.0	0.95	—	—
Met.	0	0	0	0
Activity	100%	0%	100%	0%

Assay conditions were the same as Table I.

10) H. Kanazawa, S. Uchihara, A. Ohara and M. Yoshioka, *Chem. Pharm. Bull.* (Tokyo), **18**, 195 (1970).
11) T.F. Spande and B. Witkop, *Methods in Enzymology*, **11**, 498 (1967).
12) S. Moore, D.H. Spackman and W.H. Stein, *Anal. Chem.*, **30**, 1190 (1958).
13) J.S. Spies and D.C. Chambers, *Anal. Chem.*, **21**, 1249 (1949).
14) P.D. Boyer, *J. Am. Chem. Soc.*, **76**, 4331 (1954).

mercuripapain photooxidized at pH 4.0 showed that only a tryptophan residue was oxidized and enzyme activity was completely lost, as shown in Table II. The correlation between the decrease in tryptophan content and the degree of inactivation by photooxidation at pH 4.0 is shown in Fig. 1. These findings indicate that 1 out of 5 tryptophan residues in papain may specificially relate to the enzyme activity.

Although about two tryptophan residues were oxidized at pH 4.0 by NBS, the first oxidizable tryptophan was important for enzyme activity.[6] Then, the tryptophan residues of papain and of photooxidized papain were oxidized with NBS between pH 3.0 and 6.0. The rate of oxidation by this reagent increased with the decrease of pH, and the photooxidized papain was less oxidized by one tryptophan residue than papain at all pH's tested, as shown in Fig. 2. These results indicate that a photooxidizable tryptophan residue at pH 4.0 may be identical with the first NBS-oxidizable residue.

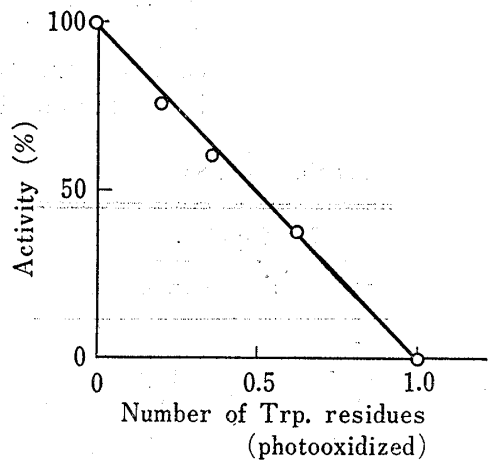

Fig. 1. The Correlation between the Decrease in Tryptophan Content and the Degree of Inactivation by Photooxidation at pH 4.0

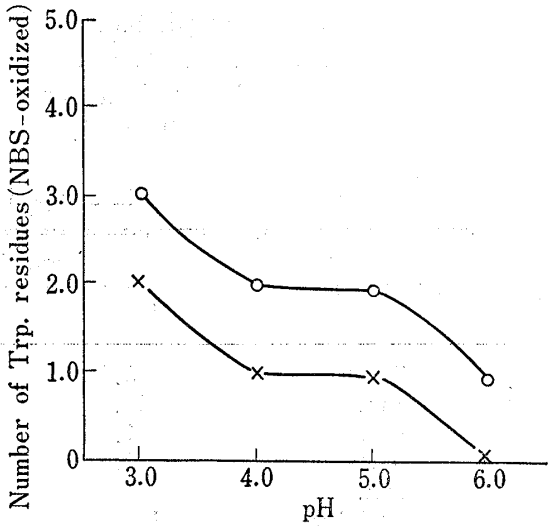

Fig. 2. Numbers of NBS-oxidized Tryptophan Residues at Various pH

○: intact papain
×: photooxidized papain at pH 4.0

Discussion

Papain was rapidly inactivated by methylene blue-sensitized photooxidation at pH 8.0 and at pH 4.0. At pH 6.0 to 8.0,[5] the rate of photo-sensitized inactivation of papain was pH dependent and about one histidine residue and two tryptophan residues per molecule of papain were lost when complete inactivation took place. Furthermore, mercuripapain was not inactivated by photooxidation, and two tryptophan residues and no histidine residue were affected in the illuminated mercuripapain (Table I). The results of photooxidation of papain at pH 6.0 to 8.0 indicate that the loss of histidine may cause the inactivation and the loss of tryptophan may not participate in the inactivation.[5] In the case of the photooxidation at pH 4.0, however, only a tryptophan residue was lost during inactivation. This tryptophan is considered to be identical with the first oxidizable residue by NBS (Fig. 2 and Ref. 1, 6). On the other hand, from the results of NBS oxidation and photooxidation at pH 8.0[1] it may be concluded that the second and the third NBS-oxidizable tryptophan residues at pH 3.0 are identical with the photooxidizable residues at pH 8.0. These results indicate that a photooxidizable tryptophan residue at pH 4.0 may be different from two photooxidizable tryptophan residues at pH 8.0.

From the results of photooxidation with proflavin at acidic pH, Jori, *et al.*[15] suggested the following: (1) Out of the five tryptophan residues present in papain, two tryptophan residues (trp-69 and trp-7) are most susceptible to photodynamic attack with proflavin at pH 6.0 when the protein is in native conformation. The oxidative alternation of these two tryptophan residues has no any great effect on the enzyme activity of papain. (2) A tryptophan residue (trp-177) must be largely buried in native papain in solution, since its indole moiety become susceptible to attack only after prior disruption of the side chains of two first oxidizable residues (trp-69 and trp-7). The intactness of this residue is essential for the catalytic action of papain. (3) However, the results of photooxidation with proflavin at more acidic pH (pH 2.4) indicate that three tryptophan residues (trp-7, trp-69 and trp-177) are most susceptible to photodynamic attack. (4) Other two residues (trp-181 and trp-26) must be deeply buried in native papain.

The results of the photooxidation at pH 6.0 with methylene blue[5] showed that about two tryptophan residues were oxidized without the loss of enzyme activity. These findings agree with the data of Jori, *et al.*,[15] though the sensitizer (methylene blue and proflavin) is different. Generally, it could be reasonably assigned that the absorbancy difference at 280—300 nm is due to the change in state of tryptophan residues.[16] The decrease in papain absorption at this wavelength begins at about pH 3.8.[15] Therefore, it is considered that no conformational change of papain occurs at pH 4.0. The results of the photooxidation at pH 4.0 with methylene blue showed that about one tryptophan residue was oxidized with the loss of enzyme activity. This residue is different from the photooxidizable residues at pH 6.0. This finding adds special interest to the susceptibility of the respective tryptophan residue to photooxidation at each pH.

The X-ray diffraction study of crystalline papain[17] has revealed that two tryptophan residues (trp-69 and trp-177) are not buried, two (trp-7 and trp-181) are partially buried, and one (trp-26) is completely buried. On the other hand, a luminescence study of papain in solution[18] has revealed that two tryptophan residues (trp-7 and trp-69) are exposed, and three (trp-26, trp-177 and trp-181) are not exposed. These findings partly disagree with the X-ray model of papain, for in the crystal, tryptophan-177 appears to be exposed. Weinryb, *et al.*[18] hypothesized the existence of some differences in the spatial geometry of papain, paticularly in the surroundings of tryptophan-177, between solution and crystals state. The data of Jori, *et al.*[15] strongly corroborate their suggestion.[18] The X-ray diffraction[17] shows that tryptophan-177 actually touches histidine-159, which is a part of the active site. Therefore, these findings indicate that tryptophan-177 may exist in the active site of papain. Our findings, particularly of photooxidation at pH 4.0 and NBS oxidation,[1,6] agree with X-ray model of papain,[17] but disagree with the findings of Weinryb, *et al.*[18] and Jori, *et al.*[15]

Based on the findings of the photooxidation with proflavin,[15] the luminescence study[18] and the X-ray model,[17] the relationship among the tryptophan residues to be affected by the photooxidation at pH 4.0 and pH 8.0[5] with methylene blue, and NBS oxidation[1,6] can be explained, as follows: Generally, in a protein molecule, only the external amino acid side chains are readily available to the NBS and the photooxidizing agent, whereas the partially or deeply buried residues often react sluggishly or does not react at all. (1) Tryptophan-177 actually touches histidine-159, which is a part of the active site, and two tryptophan residues (trp-7 and trp-69) are apart from the active SH group (cys-25) and histidine residue (his-159).[17] Therefore, it appears reasonable to hypothesize that the tryptophan residue to be photooxidized at pH 4.0 and to be first NBS-oxidized may be tryptophan-177. (2) Two tryptophan residues

15) G. Jori and G. Galiazzo, *Photochemistry and Photobiology*, **14**, 607 (1971).
16) D.B. Wetlaufer, *Adv. Protein Chemistry*, **17**, 303 (1962).
17) J. Drenth, cited in Ref. (18).
18) I. Weinryb and R.F. Steiner, *Biochemistry*, **9**. 135 (1970).

other than the first oxidizable tryptophan residue were modified by NBS oxidation at pH 3.0. These two NBS-oxidizable residues are identical with the photooxidizable residues at pH 8.0. These findings indicate that these two residues may be exposed outside the molecule in solution. Therefore, these two residues are thought to be tryptophan-7 and tryptophan-69. (3) Other two tryptophan residues were not affected by photooxidation at pH 4.0 and pH 8.0 and NBS oxidation. It is considered that these two residues are buried inside the molecule in solution. These two residues may be tryptophan-26 and tryptophan-181. (4) The two tryptophan residues other than the essential tryptophan residue were not affected by the photooxidation at pH 4.0, and the essential tryptophan residue was not affected by the photooxidation at pH 8.0. This difference between the findings of photooxidation at pH 4.0 and at pH 8.0 is not understood well at present. The susceptibility of the respective tryptophan residue to photooxidation at each pH is now under investigation.

Based on the interpretation described above, the state of tryptophan residues of papain is illustrated schematically in Fig. 3.

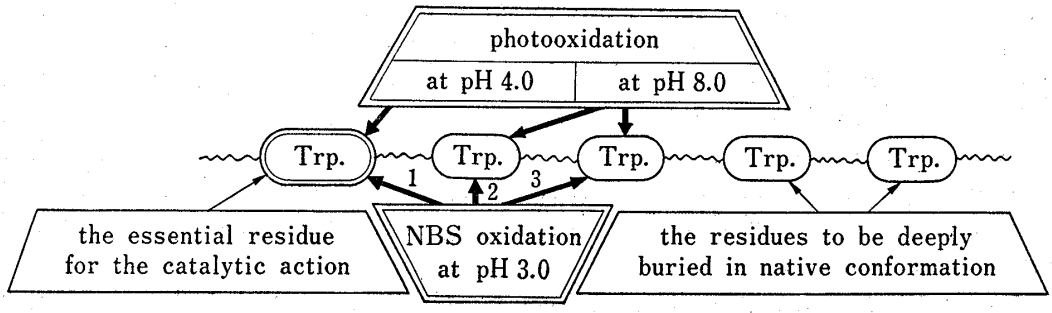

Fig. 3. The Schematic Illustration of the State of Tryptophan Residues of Papain

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