who suggested that cyclic nucleotides may act as negative feedback inhibitors of agonist-induced hydrolysis of membrane phosphatidylinositols. In support of this hypothesis, sodium nitroprusside and 8-bromo cyclic GMP also inhibited contraction and inositol monophosphate accumulation due to norepinephrine in rat aorta[22]. In addition, it has recently been suggested that the endothelium inhibits vascular smooth muscle tone and contraction through the spontaneous release of a factor which elevates cyclic GMP levels, thereby inhibiting phosphatidylinositol hydrolysis[27].

Others have suggested that phosphatidylinositol hydrolysis increases the levels of 1) phosphatidic acid, which may act as a Ca^{2+} ionophore[28], 2) inositol trisphosphate, which induces Ca^{2+} release from the sarcoplasmic reticulum and thereby induces contraction[18], and 3) diacylglycerol, which activates protein kinase C[21]. The mechanism by which cyclic GMP may act to prevent the formation of any and/or all of these potential second messengers has not been established. Nitroglycerin tolerance may be due to an inability to activate cyclic GMP-dependent protein kinase, which would phosphorylate proteins involved in the regulation of phosphatidylinositol hydrolysis.

*Acknowledgments. This work was supported by grants from the Veterans Administration, American Heart Association Southwestern Ohio Chapter, and BRSG 507 RR05-408-24 and RO1 HL 34895 from the National Institutes of Health to RMR. JKC is a recipient of a Key Pharmaceutical Company Predoctoral Fellowship. Address all correspondence to R.M. Rapoport.

1 Armstrong, P.W., and Moffat, J.A., Am. J. Med. *74* (1983) 73.
2 Parker, J.O., Fung, H., Ruggierello, D., and Stone, J.A., Circulation *68* (1983) 1074.
3 Thadini, U., Fung, H., Darke, A.C., and Parker, J.O., Am. J. Cardiol. *49* (1982) 411.
4 Bogaert, M.G., Archs int. Pharmacodyn. Ther. *172* (1968) 228.
5 Crandall, L.A., J. Pharmac. expl Ther. *48* (1933) 127.
6 Needleman, P., J. Pharmac. expl Ther. *171* (1970) 98.
7 Galvas, P.E., and DiSalvo, J., J. Pharmac. expl Ther. *224* (1983) 373.
8 Needleman, P., and Johnson, E.M., J. Pharmac. expl Ther. *184* (1973) 709.
9 Rapoport, R.M., Waldman, S.A., Ginsburg, R., Molina, C., and Murad, F., J. cardiovasc. Pharmac. (1986) in press.
10 Ignarro, L.J., and Kadowitz, P.J., A. Rev. Pharmac. Toxic. *25* (1985) 171.
11 Katsuki, S., Arnold, W.P., and Murad, F., J. cycl. Nucl. Res. *3* (1977) 239.
12 Rapoport, R.M., and Murad, F., J. cycl. Nucl. Prot. Phosphoryl. Res. *9* (1983) 281.
13 Schultz, K.D., Schultz, K., and Schultz, G., Nature *265* (1977) 750.
14 Axelsson, K.L., Anderson, R.G.G., and Wikberg, J.E.S., Acta pharmac. toxic. *50* (1982) 350.
15 Keith, R.A., Burkman, A.M., Sokoloski, T.D., and Fertel, R.H., J. Pharmac. expl Ther. *221* (1983) 525.
16 Kukovitz, W.R., and Holzmann, S., Z. Kardiol. *72* (1983) 14.
17 Wikberg, J.E.S., Axelsson, K., and Andersson, R.G.G., in: Vascular Neuroeffector Mechanisms, p. 261. Raven Press, New York 1978.
18 Berridge, M.J., and Irvine, R.F., Nature *312* (1984) 315.
19 Legan, E., Chernow, B., Parrillo, J., and Roth, B.L., Eur. J. Pharmac. *110* (1985) 389.
20 Villalobos-Molina, R., Mirna, U., Hong, E., and Garcia-Sainz, J.A., J. Pharmac. expl Ther. *222* (1982) 258.
21 Takai, Y., Kikkawa, K., and Nishizuka, Y., Adv. cycl. Nucl. Res. *18* (1984) 119.
22 Rapoport, R.M., Circ. Res. *58* (1986) 407.
23 Rapoport, R.M., and Murad, F., Circ. Res. *52* (1983) 352.
24 Berridge, M.J., Downes, C.P., and Hanley, M.R., Biochem. J. *206* (1982) 587.
25 Brown, S.L., and Brown, J.H., Molec. Pharmac. *24* (1983) 351.
26 Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J., J. biol. Chem. *193* (1951) 265.
27 Rapoport, R.M., Adv. Prot. Phosphat. *2* (1985) 263.
28 Michell, R.H., Biochim. biophys. Acta *415* (1975) 81.

0014-4754/87/0316-03$1.50 + 0.20/0

Photochemical oxidation of actinidin, a thiol protease from *Actinidia chinensis*

M. Kaneda, Y. Tomita and N. Tominaga

Department of Chemistry, Faculty of Science, Kagoshima University, Korimoto, Kagoshima 890 (Japan), 20 May 1986

Summary. Actinidin was rapidly inactivated by methylene blue-catalyzed photooxidation at pH 7.9 and 20 °C. The rate of inactivation was pH-dependent and became slower at lower pH values, suggesting the involvement of a histidine residue in the inactivation.
Key words. Actinidine, photoinactivation; protease.

Dye-sensitized photochemical oxidation of an enzyme has often been used to obtain information on the amino acid residues essential for its catalytic activity. It is well known that the photooxidation of proteins in the presence of methylene blue causes a rapid destruction of histidine and tryptophan residues and a slower destruction of tyrosine, cysteine, and methionine residues[1-4]. Martinez-Carrion studied a histidine-specific oxidation with aspartic aminotransferase[5]. Actinidin (EC 3.4.22.14) isolated from the sarcocarp of the Chinese gooseberry or kiwi fruit, *Actinidia chinensis*, is a thiol protease[6,7].

This report shows that actinidin is sensitive to dye-sensitized photochemical oxidation, and suggests that destruction of histidine is responsible for inactivation.

Materials and methods. Actinidin was isolated from the Chinese gooseberry according to the procedure of McDowal[7]. The proteinase activity of actinidin was determined by a modified Kunitz method[8] using amidated casein instead of casein as a substrate. The assay was always performed in the presence of 1 mM cysteine and 1 mM EDTA. Reaction was carried out at pH 4.0 and stopped with 10% trichloroacetic acid. Amidated casein (pI 9–10) was prepared by amidation of casein with ethylenediamine.

The pH dependence of the rate of inactivation of actinidin by methylene blue-catalyzed photooxidation was tested as follows. The reaction mixture contained 120 μM actinidin and 180 μM methylene blue, respectively, in 0.2 M phosphate-citrate buffer containing 1 mM EDTA, in a total volume of 10 ml. The reaction mixture was irradiated from a distance of 15 cm with a 100 W incandescent lamp at 20 °C. Aliquots of 50 μl were withdrawn at appropriate time intervals and used for assay of the enzymatic activity, and at the same time aliquots of 1.0 ml were withdrawn for amino acid analyses.

Photooxidized protein was freed from the reagents by passage through a column (1.5 × 31 cm) of Sephadex G-25 equilibrated and eluted with 0.1 M formic acid. The protein fractions were pooled and lyophilized. Native and photooxidized actinidin

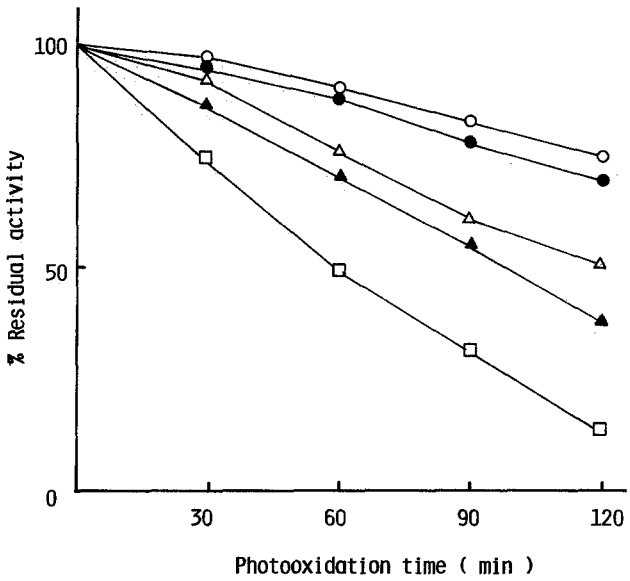

Figure 1. Rate of inactivation of actinidin by photooxidation at different pH values in the presence of methylene blue. Actinidin (120 μM) was irradiated from a distance of 15 cm with a 100 W incandescent lamp in the presence of 180 μM methylene blue at 20 °C; ○, pH 5.6; ●, pH 6.0; △, pH 6.7; ▲, pH 7.1; □, pH 7.9.

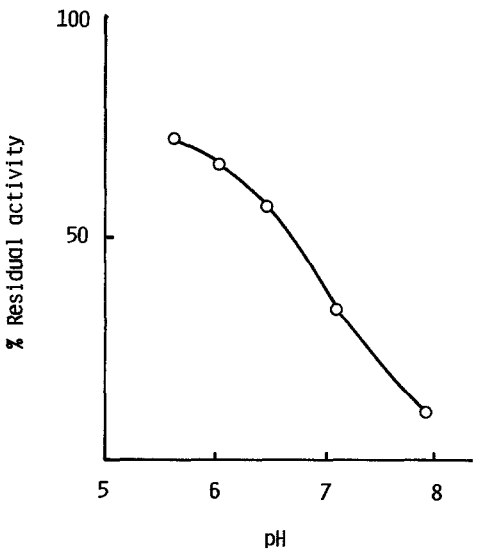

Figure 2. Effect of pH on photooxidation of actinidin. The rate of photooxidative inactivation is expressed as the percentage of that at pH 7.9 after exactly 60 min.

were hydrolyzed with 6 N HCl at 110 °C for 24 h in evacuated, sealed tubes and analyzed with an Hitachi automatic analyzer, model 034. Tryptophan was determined by the spectrophotometric method[9].

Results and discussion. As can be seen in figure 1, actinidin was rapidly inactivated by methylene blue-catalyzed photooxidation. The rate of inactivation became slower in the lower pH regions. After 100 min at pH 7.1, the enzyme retained nearly 50% of its original activity. The plot of the activity loss as a function of pH is of a sigmoidal shape and has an inflection point near pH 6.5 as shown in figure 2. This pH-dependency indicates that some amino acid residues which have a pK value of around neutrality are involved in the inactivation. Since only the photooxidation of histidine and its derivatives has been reported to show a pH-dependence similar to that observed

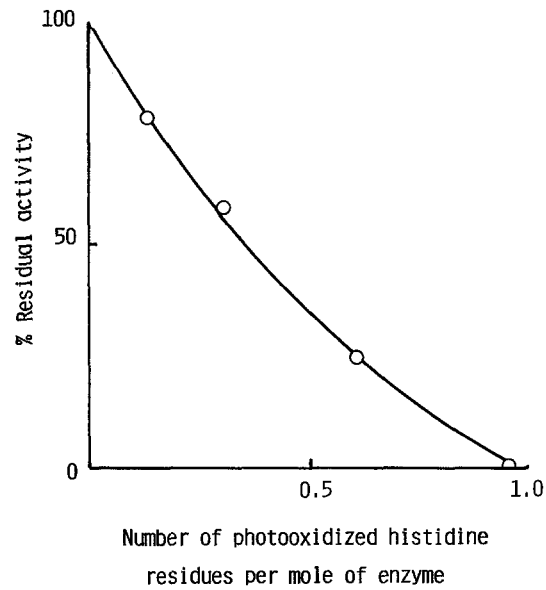

Figure 3. Relationship of activity of actinidin to the extent of photooxidation of the histidine residue at pH 7.9.

here[5, 10–12], the result strongly suggests that photooxidation of a histidyl residue of the enzyme is responsible for the activity loss. In the dark, the enzyme was quite stable in the presence of methylene blue.

After removing methylene blue from the reaction mixture by a gel-filtration, the amino acid composition of photooxidized enzyme was analyzed and compared with that for native enzyme (data not shown). The results showed that the only amino acid residue which suffered a significant change on photooxidation was histidine, and that the other residues remained almost intact within the limits of experimental error. Figure 3 shows the relationship between the histidine residue lost and the enzymatic activity remaining when the photooxidation was carried out at pH 7.9. The plotting indicates that the activity loss is completely proportional to destruction of one histidyl residue. In conclusion, the results of photooxidation experiments described here indicate that a histidyl residue is involved in the catalytic center of actinidin.

1 Weil, L., and Buchert, A. R., Archs Biochem. Biophys. *34* (1951) 1.
2 Weil, L., Buchert, A. R., and Maher, J., Archs Biochem. Biophys. *40* (1952) 245.
3 Weil, L., James, S., and Buchert, A. R., Archs Biochem. Biophys. *46* (1953) 266.
4 Weil, L., and Seibles, T. S., Archs Biochem. Biophys. *54* (1955) 368.
5 Martinez-Carrion, M., J. biol. Chem. *242* (1967) 1426.
6 Arcus, A. C., Biochim. biophys. Acta *33* (1959) 242.
7 McDowall, M. A., Eur. J. Biochem. *14* (1970) 214.
8 Kaneda, M., and Tominaga, N., J. Biochem. *78* (1975) 1287.
9 Edelhoch, H., Biochemistry *6* (1967) 1948.
10 Westhead, E. W., Biochemistry *4* (1965) 2135.
11 Weil, L., Archs Biochem. Biophys. *110* (1965) 57.
12 Freude, K. A., Biochim. biophys. Acta *167* (1965) 485.

0014-4754/87/0318-02$1.50 + 0.20/0