THE REACTION OF IODOACETATE AND BROMOACETATE WITH PAPAIN*

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The catalytic properties of papain are typical of the sulfhydryl class of enzymes, since the activity is dependent upon a free sulfhydryl group and the enzyme is inhibited by iodoacetate, p-mercuribenzoate, N-ethylmaleimide, and mercuric ions (Smith and Kimmel, 1960). A direct participation of the sulfhydryl group by formation of an acyl thiol intermediate during enzymic catalysis was suggested by Smith, Finkle and Stockell (1955). Recently, Lowe and Williams (1964) have provided spectroscopic evidence for the presence of a dithiol ester group during the reaction of papain with methyl thionohippurate, a substrate for the enzyme.

The active sulfhydryl forms a stable, inactive derivative with iodoacetamide (Finkle and Smith, 1958) or iodoacetate (Balls and Lineweaver, 1939); S-carboxymethylcysteine or S-carboxymethylcysteine sulfone was found on amino acid analysis. The position of this sulfhydryl derivative in the amino acid sequence of papain has been located in a peptide sequence 25 residues removed from the NH_2-terminus (Light et al., 1964).

In view of the above reports for the presence of a sulfhydryl group at the active center of papain, it is of interest that Yu-Kum and Chen-Lu (1963) have noted that bromoacetate and iodoacetate were

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inhibitory at pH 5 and have claimed that carboxymethylhistidine and not S-carboxymethylcysteine was formed. These workers reported that the reaction occurred in the presence of excess cysteine and that papain was partially protected against inactivation by substrates. The identification of carboxymethylhistidine was based on a two-dimensional chromatographic separation on paper by comparison with appropriate controls. It was concluded that a histidine grouping is the functional residue at the active center and the sulfhydryl group plays only a secondary role in the activity of the enzyme.

As a result of this report, the influence of excess mercaptan on the specificity of the reaction of papain with iodoacetate was reinvestigated first under the conditions previously employed (Finkle and Smith, 1958). Papain is fully active in the presence of 2,3-dimercaptopropanol (Stockell and Smith, 1957), and, under these conditions, the sulfhydryl group in papain reacted specifically with iodoacetate at pH 8.5 even when the ratio of mercaptan to iodoacetate was 7 to 1 (Table I). In the inhibited enzyme, neither carboxymethylhistidine nor ϵ-carboxymethyllysine were found after acid hydrolysis. However, the available sulfhydryl group at the active center was converted to S-carboxymethylcysteine. In a similar experiment with C^{14}-iodoacetate, the papain derivative, after dialysis against 8 M urea and then water, contained 0.52 and 0.54 moles of S-carboxymethylcysteine per mole of protein based on amino acid analyses and radioactive counting, respectively. It is clear that S-carboxymethylcysteine is the only product formed at pH 8.5 even in the presence of excess mercaptan in agreement with earlier studies. In other experiments, the yield varied from 0.5 to 0.8 residue.

When the experimental conditions of Yu-Kum and Chen-Lu (1963) were followed for the reaction of bromoacetate with papain at pH 5 in the presence of a large excess of cysteine, the reaction was again specific for the active sulfhydryl of papain (Table II). Again, it should

TABLE I

REACTION OF PAPAIN WITH IODOACETATE AT pH 8.5

Papain, 5 µmoles, was incubated in the presence of 0.005 N EDTA, 0.05 M Tris at pH 8.5, 15 µmoles iodoacetate, and 100 µmoles 2,3-dimercaptoethanol, under anaerobic conditions at 40° for 30 min. (Exp. 1) and 15 min. (Exp. 2). The initial C_1* of 2.2 decreased to zero in 30 min. A sample hydrolyzed with 6 N HCl at 110° for 20 hrs. was analyzed on the Spinco Amino Acid Analyzer. The molar ratios are based on a value of 10 for leucine. The recovery of S-carboxymethylcysteine is not corrected for losses on hydrolysis.

	Exp. 1	Exp. 2	Literature**
S-carboxymethylcysteine	0.61	0.64	1.0
Histidine	1.78	1.98	2.0
Lysine	9.00	8.52	9.0
Leucine	10.00	10.00	10.00

*C_1 is the proteolytic coefficient, the 1st order rate constant in decimal logarithms per mg protein N per ml of reaction mixture. Rate measurements were performed with benzoyl-L-arginine ethyl ester.

**Smith and Kimmel, 1960. In native papain, a single sulfhydryl group is present as a cysteine residue.

be noted that the histidine and lysine values were normal and that neither carboxymethylhistidine nor ε-carboxymethyllysine could be detected in acid hydrolysates. Essentially the same results were obtained when the reaction with bromoacetate was performed at pH 8.5. The low yield of S-carboxymethylcysteine at pH 5 was not surprising since the anionic form of the sulfhydryl group is the reactive form and the concentration of this ionic species is much lower at pH 5 than at pH 8.5 (Cecil and McPhee, 1959). It is apparent, therefore, that the functional group which reacts with iodoacetate or bromoacetate between pH 5 and pH 8.5 is the thiol group of papain. Furthermore, it may also be noted that the thiol group of papain reacts more rapidly than the thiol group of cysteine with p-mercuribenzoate (Finkle and Smith, 1958), cyanate (Sluyterman, 1964), and, as noted in this study, with

TABLE II

REACTION OF PAPAIN WITH BROMOACETATE AT pH 5

Papain, 1.34 µmoles, was incubated in the presence of 5 µmoles EDTA, 100 µmoles of citrate buffer at pH 5, 25 µmoles cysteine, and 1.4 µmoles bromoacetate at 40° for 30 min. The C_1 of 1.45 decreased to 0.0023 at 30 min. Molar ratios are based on a value of 10 for leucine. The recovery of S-carboxymethylcysteine was not corrected for losses.

	Found	Literature*
S-carboxymethylcysteine	0.36	1.0
Histidine	1.73	2.0
Lysine	9.04	9.0
Leucine	10.00	10.0

*Smith and Kimmel, 1960. See Table I.

alkylating agents. Although histidine does not participate in the reaction of haloacetates with papain, the possible involvement of this residue in the active center of papain remains a subject for further investigation.

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