STEREOCHEMICAL INVESTIGATION OF THE ACTIVE CENTER OF PEPSIN USING A NEW INACTIVATOR*

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It has been reported (Erlanger, et al, 1965) that p-bromophenacyl bromide (I) is a specific inactivator of pepsin, causing a 75-80% loss of the enzyme's ability to hydrolyze hemoglobin (Anson, 1948) and an essentially complete inhibition of its activity against carbobenzoxy L-glutamyl L-phenylalanine (Fruton and Bergmann, 1939). Supporting evidence for the specificity of the reagent and for its reaction with a functional group at or near the active center of pepsin included (a) the 1:1 stoichiometry of the reaction; (b) the ability of the virtual substrate carbobenzoxy L-phenylalanine (Sharon, et al, 1962) to competitively inhibit the inactivation reaction; and (c) the similarity between pH-rate profile of the inactivation process and that of the peptic digestion of proteins. It was shown, further, that complete reactivation could be obtained by incubation with thiophenol.

Subsequent studies (Erlanger, et al, 1966) revealed that (I) had esterified the β-carboxyl group of an unusually reactive aspartic acid residue located in a region that included one serine and two glycine residues. The reaction with an aspartic acid residue was subsequently confirmed by Gross and Morell (1966).

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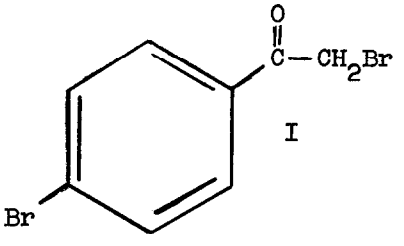
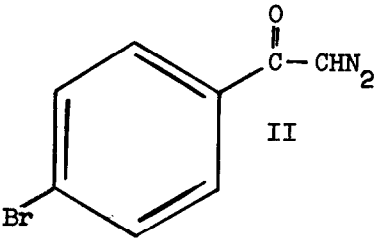
More recently a number of laboratories have reported on the inactivation of pepsin by various diazo compounds in the presence of Cu^{++} (Rajagopalan et al, 1966; Delpierre and Fruton, 1966; Hamilton, et al, 1967). In all cases reported, no peptic activity could be detected by hemoglobin assay or by assay with synthetic substrates. These findings have stimulated us to investigate the nature of the inactivation process mediated by (I), and to see how it differed from the action of the diazo compounds. Since there is good evidence that the amino acids affected by both types of inactivators are part of or near the active center of pepsin, information about the topography of the active center was also sought. Therefore, a new diazo-type inactivator was designed bearing the same alkylating group as (I). This new inactivator is α-diazo-p-bromoacetophenone (II).



I



II

Although (I) and (II) might be expected to bind to the same part of the active site of pepsin, it is not likely that they would substitute the same amino acid residue since (I) is an electrophilic reagent and (II) reacts primarily as a nucleophile (Zollinger, 1961). However, it can be reasonably concluded that, if two different functional groups are involved, they are near one another, although not necessarily on the same primary sequence.

It was, in fact, found that (I) and (II) reacted with two different amino acid residues. Furthermore, the active aspartic acid residue substituted by (I) was shown not to play an essential role in the catalytic

mechanism of pepsin. Substitution of this residue, however, interfered stereochemically with the enzyme-substrate interaction.

Materials and Methods

All pepsin preparations were Worthington 2X-crystallized.

Analyses were carried out by Joseph Alicino, Metuchen, New Jersey.

α-Diazo-p-bromoacetophenone (II) - p-Bromobenzoyl chloride (0.01 mole) was added to an ether solution of diazomethane at +5° prepared by the action of 70% KOH on 3.4 g of N-nitrosomethyl urea. The solution was allowed to stand for 16 hours at room temperature. The yellow crystalline precipitate was collected and dried *in vacuo*; yield, 1.73 g, 72%, m.p. 120.5-121.5° (dec.). Its infrared spectrum in chloroform showed the typical α-diazoketone band at 4.71 microns. Recrystallization from n-butanol raised the m.p. to 123-124° (dec.). Erickson, et al (1951) reported m.p. 123.5-124°. The compound was further characterized by conversion in almost quantitative yield to p-bromophenacyl acetate by reaction with acetic acid; m.p. after crystallization from ether-petroleum ether 85-86°; Erickson, et al (1951), reported the same melting point. The ultraviolet spectrum of (II) in methanol has maxima at 229 mμ (ϵ max, 15,000) and at 259 (ϵ max, 16,500). Upon reaction with pepsin, a peak is exhibited at 260 mμ only, which is characteristic of the p-bromophenacyl moiety and corresponds to the 260 mμ maximum (ϵ max, 16,300) of (I). Thus, (II) does not undergo the Wolf rearrangement prior to its reaction with pepsin but takes part in a direct alkylation reaction (cf. Zollinger, 1961).

Inactivation of Pepsin by (II) - Pepsin (1.0 g, 2.75×10^{-5} mole) was dissolved in 340 ml of 0.006 M acetate buffer, pH 5.0. To this solution was added 75 ml of 0.01 M CuCl (7.5×10^{-4} mole) with stirring. After ten minutes, 24.1 mg (1.07×10^{-4} mole) of (II) in 25 ml of ethyl alcohol was added. The blue solution remained clear and was allowed to stand for 24 hours at 27°. An aliquot withdrawn and assayed against hemoglobin showed complete inactivation. The product was dialyzed for 25 hours against distilled water in the cold room and then lyophilized. The dry product (blue

in color) was extracted 5 times with 500 ml portions of anhydrous ethyl ether, each extraction requiring 24 hours of contact in the cold room. The last extract showed no absorption at 260 mμ. Yield, 0.892 g.

Treatment of Pepsin Inactivated by (I) with (II) - Pepsin (1 g, 2.75×10^{-5} mole), was dissolved in 320 ml 0.0033 N HCl. p-Bromophenacyl bromide (16 mg, 5.7×10^{-5} mole), in 10 ml of methanol was added with stirring (final pH 3.2). Stirring was terminated after two hours and the reaction allowed to proceed at 25° for 22 hours. An aliquot was assayed using hemoglobin as substrate and was found to be 78% inactivated. The pH of the solution was adjusted to 5.0 with 5% $NaHCO_3$. Then 20 ml of 0.1 M acetate buffer pH 5.1 and 75 ml of 0.01 M $CuCl_2$ were added with stirring. After 15 minutes, 24.1 mg of (II), 1.07×10^{-4} mole, in 25 ml of ethanol were added and stirring was continued for 22 hours at 27°. Evolution of nitrogen was apparent. Assay of an aliquot against hemoglobin showed no activity.

The solution was then dialyzed in the cold room for a period of 24 hours against 3 changes of distilled water. It was then lyophilized. The dry product was exhaustively washed with ether as described above, before analysis for bromine content.

Enzyme kinetic experiments were run according to Silver, et al, 1965.

Acetyl L-phenylalanine L-tyrosine was a product of Cyclo Chemical Corp.

Results and Discussion

Incubation of (II) (2.43×10^{-4}M) at 27° with pepsin (6.25×10^{-5}M) at pH 5.0 (0.0046 M acetate buffer) in the presence of 0.0017 M $CuCl_2$ and 10% ethanol resulted in complete inactivation of pepsin (by hemoglobin assay) with a reaction half-time of about 10 minutes. A suitable pepsin control lost no activity. Following dialysis and lyophilization, the inactivated product was extracted exhaustively with ether until no 260 mμ absorbing material could be detected in the extract. The product was then analyzed for bromine; calculated for one gram-atom per mole of enzyme (MW 36,400), 0.219%; found, 0.202%. The bromine analysis was confirmed by an examination of the difference spectrum of inactivated pepsin vs. pepsin in aqueous solu-

tion. Thus, inactivation by (II) resulted from a reaction having a 1:1 stoichiometry. Unlike pepsin inactivated by (I), no reactivation by thiophenol could be observed.

The fact that (II), unlike (I), caused complete inactivation of pepsin and that reactivation could not be induced by thiophenol suggested that (I) and (II) did not react with the same functional group on the pepsin molecule. This was established with certainty by showing that pepsin substituted with (I) was still able to react with (II). Pepsin (1 g, 2.74×10^{-5}mole) was allowed to react with (I) (0.016 g, 5.7×10^{-5} mole) according to the previously reported procedure (Erlanger, et al, 1965). Assay with hemoglobin showed a 78% decrease in activity. The pH of the solution was then adjusted to pH 5.0 and (II) (0.0241 g, 1.07×10^{-4} mole) in 25 ml ethanol was added. After incubation at 25° for 22 hours, assay with hemoglobin substrate showed complete loss of activity. The product was isolated by dialysis and lyophilization, extracted exhaustively with ether, as described above, and submitted for bromine analysis; calculated for two gram atoms per molecule of pepsin (MW 36,400) 0.44%; found, 0.38, 0.41%. The data are in agreement with the existence of two p-bromophenacyl moieties on the pepsin molecule. Difference spectrum studies confirmed these results.

The above results prove that (I) and (II) react with different amino acid residues at or near the active site of pepsin. Furthermore, they indicate (a) that (II), because it inactivates pepsin completely, probably reacts with a functional group that participates directly in the catalytic mechanism; and (b) that the aspartic acid residue substituted by (I) does not play an essential role in catalysis.

With respect to (b), kinetic studies using acetyl L-phenylalanine-L-tyrosine as substrate (Silver, et al, 1965), have revealed that pepsin inhibited by (I) is still active but that the K_m of the enzyme-substrate interaction is $10.5 \pm 1.0 \times 10^{-3}$M as compared with 1.6×10^{-3}M for pepsin (Silver, et al, 1965 reported 1.68×10^{-3}M); k_3 was practically unchanged. Therefore,

substitution of the aspartic acid residue interferes stereochemically with the enzyme-substrate interaction. An analogous situation has been observed with chymotrypsin by modification of a methionine residue (Koshland, et al, 1962; Lawson and Schramm, 1962).

We are now proceeding with studies designed to identify the amino acid residue substituted by (II).

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