A CHEMICAL INVESTIGATION OF THE ACTIVE CENTER OF PEPSIN*

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It was recently reported (Erlanger, et al, 1965) that p-bromophenacyl bromide (I) was a specific inactivator of pepsin. Evidence that I reacted with an amino acid residue at or near the active center of pepsin includes the following: (a) Inactivation resulted from a stoichiometric 1:1 reaction between I and pepsin. (b) The inactivation process could be competitively inhibited by substrates of pepsin including carbobenzoxy L-phenylalanine (Sharon, Grisaro and Neumann, 1962) and acetyl L-phenylalanine L-tyrosine (Silver, Denburg and Steffens, 1965). (c) The pH-rate profile of the inactivation process was similar to that for the peptic digestion of proteins (Pope and Stevens, 1951). (d) More than 20 reagents closely related to I in structure and chemical reactivity failed to inactivate pepsin.

This paper reports on the site of attachment of I and the identity of some neighboring amino acids.

p-Bromophenacylpepsin was prepared from Worthington 2x-crystallized pepsin (lots 697, 710 and 711) as previously described (Erlanger, et al, 1965) and, after lyophilization, was exhaustively washed with ether to remove excess I. Two and one half grams were suspended in 70 ml of water, the pH adjusted to 7.0 with sodium bicarbonate and 50 mg of Worthington 2x-crystallized trypsin and 50 mg of 3x-crystallized α-chymotrypsin were added. The solution was maintained at pH 7.0 for 19 hours at 27° using a pH-stat, and then lyophilized.

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A portion of the digest was digested further by pronase (Calbiochem) at pH 7.0, 20° for 24 hours using a ratio of inactivated pepsin to pronase of 50 to 1. The pronase digest was then allowed to react at pH 1.5 with an excess of either 2,4 dinitrophenylhydrazine (II) or 4-nitrophenylhydrazine-2-sulfonic acid (III) in order to convert the p-bromophenacyl moiety into a colored hydrazone that could be identified during subsequent procedures. In the case of treatment with III, a sulfonic acid group was also introduced, thus simplifying the task of separating active site peptides from the rest of the peptide fragments.[1] Repeated high voltage electrophoresis at pH 1.9 and 5.6 and paper chromatography in various systems yielded a number of yellow peptides, the smallest having the composition (Gly_2, Asp, Ser, Glu).

The attachment of I to pepsin was shown to be via an ester linkage as evidenced by the following findings: (a) Tryptic-chymotryptic digests of p-bromophenacylpepsin and of pepsin were tested for the presence of ester groups using the Fe^{+++}-hydroxamate reaction (Hestrin, 1949).[2] Native pepsin unexpectedly showed two ester or ester-like groups. However, one additional group was found in p-bromophenacylpepsin. Molar color yields were determined using carbobenzoxyaspartic acid β-benzyl ester as the standard. (b) Treatment of pronase digests of p-bromophenacylpepsin and of pepsin with N-(γ-aminopropyl)-N'-methylpiperazine in methanol resulted in the formation of new highly basic peptides (as shown by high voltage electrophoresis at pH 5.6) indicating that aminolysis had occurred. Two of the peptides were found in both pepsin and p-bromophenacyl pepsin but an additional set of peptides were in the latter.

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[1] In this case, it was necessary to remove all phosphopeptides before reaction with III to avoid possible contamination in electrophoretic separations of the active site peptides. This was done by passing the digest through a Dowex-2 column. Elution with 0.015 N HCl removed all peptides except the phosphopeptides. This was ascertained by electrophoresis at pH 1.9 of a sample of the eluate. Negatively charged peptides present before passage through Dowex-2 were no longer present in the eluate.

[2] If prior enzymic digestion were not carried out precipitation of protein occurred during the analytical procedure.

Since the smallest fragment isolated contained both glutamic and aspartic acids, further work was necessary to identify the residue that was esterified by I. The procedure of Blumenfeld and Gallop (1962) was applied. In this procedure, carboxylic ester groups are converted to amines by Lossen rearrangement of the N-(2,4-dinitrophenyl) hydroxamate. The appearance of 2,3-diaminopropionic acid in an acid hydrolysate of p-bromophenacylpepsin so treated indicated that the site of attachment of I was the β-carboxylate of an aspartyl residue. Moreover, in agreement with the results of the Fe^{+++}-hydroxamate test and the aminolysis experiment, 2,4-diaminobutyric acid was present in hydrolysates of both native and inactivated pepsin subsequent to Lossen rearrangement. Neither of these amino acids was observed if the Lossen rearrangement were omitted.

We conclude, then, that the inactivation of pepsin by I occurs as a result of a reaction with an aspartic acid residue in a region of the active site with the composition (Gly_2, Asp, Ser, Glu). One cannot help but note the striking resemblance to the amino acid contents of portions of the active sites of the so-called "serine esterases" such as chymotrypsin, aliesterase, acetylcholinesterase, etc. (P. Desnuelle, 1960). Yet pepsin differs markedly from them with respect to pH optimum and its susceptibility to specific inactivators such as diisopropylfluorophosphate and diphenylcarbamyl chloride (Erlanger and Cohen, 1963).

Our findings also indicate that commercial preparations of 2x-crystallized pepsin contain possibly two ester-like linkages involving γ-carboxylates of glutamic acid. We are studying the nature and significance of these linkages.

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