An Active Site Peptide from Pepsin

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WE report the inactivation of pepsin by a specific irreversible inhibitor of substrate-like structure, the isolation of a heptapeptide containing the residue to which the irreversible inhibitor is attached, and the identity of what is almost certainly one of the catalytic functionalities of this enzyme.

The design of what Baker[1] has called an active-site-directed irreversible inhibitor is based upon two considerations: the substrate specificity of the enzyme, and the chemical nature of the enzymic group it is intended irreversibly to modify. Studies of the pepsin-catalysed hydrolysis of model dipeptide substrates have shown that there is an absolute requirement that the amino-acid residues on either side of the bond that is cleaved must be of the L-configuration, and of the good substrates reported at least one of these residues is aromatic.[2] Accordingly our inhibitor was derived from L-phenylalanine. For uncharged model dipeptide substrates, the enzyme shows a bell-shaped pH-activity dependence, with apparent pK_a values of 1·0 and about 4·8.[3] Although deductions concerning the identity of catalytic functionalities of enzymes from pK_a data alone are dangerous, the remarkably low pH optimum of this enzyme makes it very probable that one (if not two[4]) carboxyl group is involved in the catalytic mechanism. Some of the most effective reagents for carboxyl group modification in proteins are diazo-compounds, and for this reason the inhibitor chosen for evaluation was *N*-diazo-[1-^{14}C]acetyl-L-phenylalanine methyl ester.

Since this work was started, parallel studies using specific[5–7] or less specific[8–10] diazo-compounds as pepsin reagents have been reported, though neither the residues attacked nor any peptide containing them, have yet been identified. Using a different type of reagent (*p*-bromophenacyl bromide), it has been found that an aspartic acid residue is modified,[11,12] though more recent work by Erlanger and his group[10] has cast doubt on the functional necessity of the aspartic acid residue labelled by the bromo-ketone. Indeed, Erlanger has suggested[10] that his diazo-reagent (which is less specific for the enzyme than that used in the present work) "reacts with a functional group that participates directly in the catalytic mechanism".

N-Diazo[1-^{14}C]acetyl-L-phenylalanine methyl ester was prepared by the diazotisation of [1-^{14}C]-glycyl-L-phenylalanine methyl ester hydrobromide, and had m.p. 126—128°, $[\alpha]_D^{20}$ + 186° (*c* 1 in chloroform). This material (1·8 mM) reacts very rapidly with pepsin (60 μM) at pH 5·0, 20°, in the presence of cupric acetate (2·3 mM), and under these conditions enzymic activity (using hemoglobin as the substrate[13]) was undetectable after 10 min. The rates of loss of activity with respect

to hemoglobin and to the model substrate *N*-acetyl-L-phenylalanyl-L-phenylalaninamide, were the same within experimental error (*cf.* refs. 10, 11). After removal of the excess of reagent using a column of Sephadex G-25, the radioactivity of the inactivated enzyme indicated that 1·00—1·05 mole of reagent had reacted with 1 mole of pepsin (assuming a value for $E^{280}_{0\cdot1\%}$ of 1·47 for the latter). The competitive inhibitor *N*-acetyl-L-phenylalanine ethyl ester was found to protect the enzyme from inactivation by the diazo-reagent. At a concentration of *N*-acetyl-L-phenylalanine ethyl ester of 14·4 mM, 4 M-pepsin, and 1·8 mM-inhibitor, the rate of loss of enzymic activity at 0° was approximately halved. This indicates (though it cannot prove) that the reaction between pepsin and the diazo-reagent involves the active site of the enzyme. Due to the instability of the reagent in aqueous solutions at pH values below 4, and of pepsin at pH values above 5·5, we did not attempt to determine the pH dependence of the inactivation reaction.

The major difficulties encountered in the isolation of the labelled peptide sprang from the lability of the enzyme-inhibitor bond. Consistent with the expected existence of an ester link between a carboxyl group on the enzyme and the reagent, it was found that the label was lost from the inactivated enzyme at pH values above 7. Moreover, it appeared that after the protein had been denatured and digested, the label was *more* easily lost from the resulting peptide than from the intact protein. The pH was therefore kept below 5 in all operations subsequent to digestion. It was also found that the labelled peptide lost its label on freeze-drying in the presence of inorganic salts. The use of DEAE-Sephadex and a salt gradient in the eluant, which initially showed promise, was therefore ruled out. The following procedure was used to obtain and purify the labelled peptide.

An aqueous solution of denatured, inhibited enzyme (approx. 15 mg./ml.) was digested with native pepsin (5% w/w of inhibited enzyme) at pH 3·0, 35°, for 12 hr. This digest was subjected to chromatography on Sephadex G-25 with water as the eluant. Two radioactive peaks eluted from the column: the first emerged close to the void volume of the column and was assumed to be largely undigested material; the second peak eluted at about twice the void volume, and contained between 30 and 50% of the total radioactivity in the inhibited enzyme. The material comprising this second peak was further purified by vertical electrophoresis at pH 3·5 (pyridine–acetate buffer), from which one major radioactive spot (containing 80—90% of the radioactivity applied to the paper) was obtained. This material was further purified by electrophoresis at pH 1·9 (acetate–formate buffer), followed by descending paper chromatography using t-butyl alcohol : methyl ethyl ketone : water (2 : 2 : 1 v/v) as the eluant. In each of the final two stages, a single radioactive spot was obtained. At each stage of purification on paper, a guide strip was cut from the edge of the paper, and developed with ninhydrin–cadmium reagent.[14] The last two stages of purification (electrophoresis at pH 1·9, and paper chromatography) were sometimes performed in the reverse order. However, since the unlabelled peptide migrates at pH 1·9 rather similarly to the labelled peptide, this resulted in a slightly low yield of phenylalanine from the label, after acid hydrolysis (see the Table).

From exploratory analyses it became clear that the peptide contained both Val and Ile (which are released slowly on acid hydrolysis) and Ser and Thr (which are partially destroyed on acid hydrolysis). Accordingly, a single batch of purified peptide was divided into three parts and hydrolysed for 22, 48, and 94 hr. The results are shown in the Table. From this it is clear that the peptide has

TABLE

*Amino-acid analyses of the labelled peptide**

Amino-acid	Hydrolysis time (hr.) 22	48	94	Integral values (extrapolated)
Asp	1·0	1·0	1·0	1
Thr	1·75	1·68	1·59	2
Ser	1·08	1·04	0·98	1
Gly	1·15	1·31	1·28	1
Val	0·32	0·73	0·95	1
Ile	0·37	0·73	0·92	1
Phe	0·79	0·87	0·89	1

* Beckman-Spinco analysers were used. Values are normalised to Asp = 1·0. No other amino-acids were present in measurable quantity.

the composition Asp_1, Thr_2, Ser_1, Gly_1, Val_1, Ile_1. On the basis of the extreme slowness with which the peptide colour appears on treatment with ninhydrin–cadmium reagent, it seems probable that Val or Ile is the *N*-terminal residue. As a check experiment, the lability of the radioactive label was turned to good account by running a 'diagonal' in the manner of Hartley and his co-workers.[15] A sample of the second radioactive peak from Sephadex was subjected to electrophoresis at pH 3·5 in one dimension, and the strip was then treated with a saturated aqueous solution of triethylamine. After electrophoresis at pH 3·5 perpendicular to the original direction, one major ninhydrin-positive spot was observed off the diagonal, in exactly the position expected for the peptide with a fractional additional negative charge (at pH 3·5) due to the unmasking of the β-carboxyl group of an aspartic acid residue. This is taken as additional evidence that the bond between label and peptide is an ester on the aspartic acid β-carboxyl group. Moreover, when the purified labelled peptide was treated on paper with aqueous triethylamine and re-run electrophoretically at pH 1·9, the radioactivity remained at the origin, and amino-acid analysis of the single ninhydrin-positive spot (after elution and 22 hr. hydrolysis) was in reasonable agreement with the 22 hr. hydrolysis in the Table, with the exception that *no* phenylalanine was detectable. While the possibility of other peptides containing the radioactive label cannot be ruled out, the yields of the peptide described here make it certain that this is an important, and possibly unique, site of attack of the enzyme by the inhibitor.

In summary, we suggest that the inhibitor *N*-diazoacetyl-L-phenylalanine methyl ester reacts with a single aspartic acid residue at the active site of pepsin. This residue is part of a peptide of composition Asp,Thr_2,Ser,Gly,Val,Ile. Sequence studies are in progress.

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