ISOLATION OF A SPECIFIC ACYL-CHYMOTRYPSIN INTERMEDIATE

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It is generally accepted that the catalytic pathway of esterolytic and proteolytic "serine Enzymes" (chymotrypsin, trypsin, acetylcholineesterase) involves a three step mechanism: rapid establishment of an enzyme-substrate complex, formation of an acyl-enzyme intermediate, and deacylation (by hydrolysis) to free enzyme and acid. The acyl enzyme hypothesis is based mainly on kinetic data, on catalyzed transfer reactions or on spectrophotometric shifts occurring during the enzymatic hydrolysis of compounds like p-nitrophenyl acetate and cinnamoyl imidazole (Bender and Kezdy, 1965). Acetyl--chymotrypsin has been prepared by reacting the enzyme with p-nitrophenyl acetate (Balls and Wood, 1956) and it was shown that the acetyl group was linked to the active serine (Oosterbaan and van Andrichem, 1958). Such direct evidence has not yet been demonstrated with specific substrates, which are hydrolyzed 10^3 or 10^4 fold faster than p-nitrophenyl acetate.

In this study an acyl-enzyme compound was prepared by rapid denaturation of chymotrypsin after prior incubation with the chromogenic substrate N-acetyl--3-nitrotyrosine ethyl ester in acid solution. After digestion of the acyl--chymotrypsin derivative with pepsin and separation of the peptides by high voltage paper electrophoresis, the nitrotyrosyl group was found attached to a peptide which contained the active serine residue.

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EXPERIMENTAL

Preparation of N-acetyl-3-nitrotyrosyl-chymotrypsin

Chymotrypsin (Worthington 3 x crystallized, 20 mg) was dissolved in HCl (0.001 M, 10 ml) and incubated at 20° with N-acetyl-3-nitrotyrosine ethyl ester (3 mg) and after 5 minutes an equal volume of 10 percent trichloroacetic acid was added. The precipitated protein was centrifuged and washed three times by resuspension in 5 percent trichloroacetic acid, after which no traces of the nitrotyrosine derivative could be detected in the supernatant solution. A part of the precipitate was dissolved in sodium hydroxide, 0.1 N, and from the absorbancy due to protein at 280 mμ and that due to the nitrophenolate chromophore at 420 mμ it was calculated that 0.5 nitrotyrosyl groups were bound per protein molecule.

Isolation of a nitrotyrosyl-peptide

A pepsin hydrolyzate of nitrotyrosyl-chymotrypsin (10 mg protein derivative, 1 mg pepsin, 2 ml 5 percent formic acid, 37°, 16 hours) was applied as a 20 cm band on Whatman No. 1 paper and the peptides were separated by high voltage electrophoresis (60 volts per cm, 40 min, 25°) using pH 6.5 buffer (pyridine acid: water: 25:1:25 v/v) and xylene as coolant.

The nitrophenolate chromophore was easily detected by exposing the dried electrophoretogram to ammonia vapour. Two yellow zones were thus observed, one cationic (2.2 cm from neutral) and one anionic (15.1 cm from neutral). The mobility of the anionic yellow zone was the same as that of N-acetyl-3-nitrotyrosine run as marker on the same paper. Also, the anionic yellow zone did not coincide with any coloured zone on a guide strip developed with Cd-ninhydrin reagent, indicating that the nitrotyrosyl group is not linked to a peptide. The cationic yellow band coincided with a zone that gave a brown colour with Cd-ninhydrin reagent and fluoresced under a U.V. lamp. This zone

was eluted from the paper, the dried eulate hydrolyzed (6 N HCl, 110°, 24 hours), and the hydrolyzate analyzed in a Beckmann-Spinco Amino Acid Analyzer. Free 3-nitrotyrosine was also run in the amino acid analyzer and it appeared 12 minutes after the phenylalanine peak (50 cm column, 70 ml per hour).

RESULTS

An initial attempt to trap the nitrotyrosyl enzyme intermediate at pH 7 by denaturation was unsuccessful. However, at pH 2.5 a denatured stable nitrotyrosyl chymotrypsin could be prepared in about 50 per cent yield.

Identification of the locus of attachment of the nitrotyrosyl residue to the enzyme was based on the finding by Brown and Hartley (1966) that a double cystine peptide containing the active center serine can be easily detected and purified by high voltage electrophoresis at pH 6.5 of a pepsin digest of chymotrypsin. We therefore digested the denatured nitrotyrosyl - -chymotrypsin with pepsin and separated the peptides under the conditions reported by Brown and Hartley (1966) and indeed on exposure of the paper electrophoretogram to ammonia vapour, the nitrotyrosyl-group was detected as a yellow zone which otherwise appeared the same as the active center peptide (e.g. electrophoretic mobility, ninhydrin colour, fluorescence under a U.V. lamp). It should be pointed out that because of its large size for a single positive charge, the active center peptide is well resolved from all other peptides in the one dimensional fingerprint of pH 6.5. The proposed structure of the nitrotyrosyl-peptide is shown in Fig. I. It was deduced from the amino acid composition (Table 1) of the peptide and the amino acid sequence of chymotrypsin (Hartley, 1964), and pairing of disulfide bonds reported by Brown and Hartley (1963). The observed amino acid composition is in good agreement with that expected for the structure in Fig. 1. The somewhat low

TABLE 1

The amino acid composition of a nitrotyrosyl-peptide isolated for a pepsin digest of nitrotyrosyl-chymotrypsin and the amino acid composition expected for the active serine peptide structure given in Fig. 1.

Amino Acid	Composition		Amino Acid	Composition	
	Observed	Expected		Observed	Expected
Aspartic acid	2.4	2	Isoleucine	0.0	0
Threonine	6.7	7	Leucine	1.5	2
Serine	6.2	6	Tyrosine	0.8	1
Glutamic acid	0.1	0	Phenylalanine	0.3	1
Proline	2.0	2	Nitrotyrosine	0.3	1
Glycine	8.6	9	Tryptophan	+	2
Alanine	2.8	3	Lysine	1.9	2
Half-cystine	3.5	4	Histisine	0.0	0
Valine	3.5	3	Arginine	0.0	0
Methionine	1.0	1			

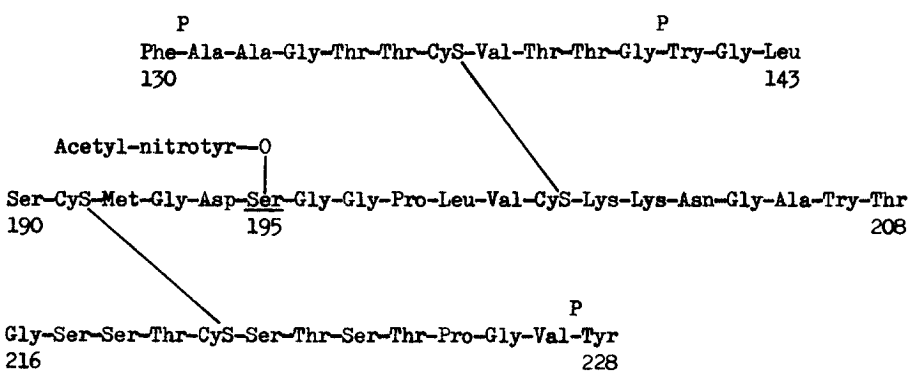

Fig. 1. Proposed structure of N-acetyl-3-nitrotyrosyl-peptide based on the sequence and numbering given by Hartley (1964) and the S-S pairing according to Brown and Hartley (1963, 1966). P designates partial splitting by pepsin.

values of threonine, glycine, leucine, tyrosine and phenylanine are probably due to partial splitting by pepsin. The somewhat high values of aspartic acid and valine are difficult to explain, but these were also observed by Brown and Hartley (1966). Half-cystine is partially destroyed by acid hydrolysis. The low recovery of nitrotyrosine can be ascribed to partial labeling of chymotrypsin and to hydrolysis during the pepsin digestion, since free acetyl--nitrotyrosine was also found in the fingerprint of the pepsin hydrolyzate. There is therefore little doubt that the nitrotyrosyl group is linked to the active center peptide, probably on the active serine as indicated in Fig. 1.

As there are many other serine and threonine residues which could be sites of acylation, it would be desirable to degrade the peptide to smaller fragments so as to have unambiguous proof for the site of attachment. An attempt to do this by digestion with subtilisin failed because the nitrotyrosyl group was also completely removed as N-acetyl-nitrotyrosine. We are searching for ways to degrade the nitrotyrosyl-peptide which will leave the chromophore attached.

DISCUSSION

A stable acetyl-chymotrypsin has been isolated, after treating the enzyme with p-nitrophenyl acetate at pH 5 (Balls and Wood, 1956). By subjecting this acetyl-enzyme to proteolytic digestion Oosterbaan and van Andrichem (1958) were able to identify the site of acylation as serine 195.

Since specific substrates of chymotrypsin, like N-acetyl tyrosine ethyl ester or N-acetyl-3-nitrotyrosine ethyl ester (a somewhat better substrate), are normal esters but nevertheless are hydrolyzed 10^3-10^4 fold faster than active esters such as p-nitrophenyl acetate or cinnamoyl imidazole, it was reasonable to question whether the same catalytic pathway exists in each case. Several lines of evidence based on kinetics or spectral changes of the enzyme reaction with specific substrates strongly support the acyl enzyme hypothesis.

For instance, when a series of tryptophane or phenylalanine esters reacted with chymotrypsin the rate of hydrolysis was the same, irrespective of the alcohol moiety, suggesting that a common acyl intermediate was formed, the deacylation of which was rate controlling (Zerner, Bond and Bender, 1964).

Incubation of chymotrypsin with its substrates in the presence of nucleophiles like alcohol, hydroxylamine and H_2O^{18} gave rise to catalyzed transfer reactions where esters (Bender and Glasson, 1960; Bender et al., 1964) hydroxamates (Bernhard et al., 1960; Epand and Wilson, 1963; Bender et al., 1964) and O^{18} labeled acids (Sprinson and Rittenberg, 1951) were formed.

It has been demonstrated recently that when a specific substrate N-acetyl-L-tryptophan ethyl ester reacted with chymotrypsin, a biphasic spectral change took place which is consistent with acylation-deacylation mechanism (Kezdy, Clement and Bender, 1964).

In this work we tried to find direct evidence for the existence of the specific acyl enzyme, by isolating it. Our attempt to trap the N-acetyl nitrotyrosyl enzyme intermediate by denaturation at neutral pH failed. Since chymotrypsin hydrolyzes N-acetyl tyrosine ester substrates very rapidly at this pH (about 200 substrates molecules are cleaved by an enzyme molecule per second) and because of the relatively high concentration of enzyme needed for the trapping experiment the substrate was consumed before denaturation took place. However, under acid conditions (pH 2-3) the lifetime of the acyl intermediate compound should be in the order of a minute and thus the acetyl--nitrotyrosyl enzyme was trapped by denaturation and identified by its yellow colour.

The results presented in this paper give direct evidence that an acyl--enzyme intermediate is formed with a good substrate, although this has only been demonstrated under conditions well below that of optimum activity. However, as the pH profile of the chymotrypsin reaction rate shows that from

pH 2 to 8 the rate is dependent on a single basic group of pK 6.8 it is reasonable that the same mechanism operates at optimal pH as in lower pH values.

The exact site of acylation on the enzyme has not yet been determined, due to the lability of the nitrotyrosyl-peptide towards further fragmentation. Attempts are being directed to overcome these difficulties and identify the site of acylation. But as the nitrotyrosyl group is attached to a peptide containing the "active serine 195" it is probable that this is the site of the tyrosyl linkage.

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