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SPECIFICITY OF NITROTRYPSIN AND SOME OF ITS OTHER CHARACTERISTICS

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The activation of nitrotrypsinogen by trypsin proceeds very slowly and is not paralleled by autoactivation. Nitrotrypsinogen does not react with dinitrofluorobenzene whereas nitrotrypsin reacts giving rise to DNP-isoleucine and DNP-valine. Nitrotrypsin when assayed with the B-chain of oxidized insulin as substrate shows the same specificity as trypsin and its nonspecific chymotryptic activity is not affected by nitration.

We have observed earlier[1,2] that nitrotrypsin prepared by the nitration of trypsin differs in its activity from trypsin obtained by the activation of nitrotrypsinogen. In the meantime, Vincent and coworkers[3] studied in more detail the enzymatic properties of trypsin after its nitration. Our present study has been aimed at the comparison of the specificity of cleavage of a protein substrate (the B-chain of S-sulfoinsulin) by nitrotrypsin and trypsin. At the same time some of our observations are confronted with the data of French authors.

EXPERIMENTAL

Material and Methods

Trypsinogen, trypsin, tetranitromethane, and the nitration of trypsin have been described earlier[1]. The nitration of trypsinogen and the characteristics of nitrotrypsinogen have been reported elsewhere[2]. The B-chain of S-sulfo-insulin was electrophoretically homogeneous (on paper in dilute formic acid), had one N-terminal end group (phenylalanine), and its amino acid analysis corresponded to the composition of the B-chain. *The determination of tryptic activity* was carried out using two substrates: benzoyl-L-arginine ethyl ester (BAEE), by titration with 0·1M-NaOH in the autotitrator (Radiometer TTTl) at pH 8·0 and 25°C, or tosyl-L-arginine methyl ester (TAME), spectrophotometrically (Zeiss-Opton) at pH 8·0 and 25°C. *Activation of nitrotrypsinogen.* The solution of nitrotrypsinogen (concentration 15 mg/ml, at pH 3·0) was mixed with 2M solution of $CaCl_2$ and the pH of the mixture was adjusted to pH 8·0 by 0·5M solution of tris-(hydroxymethyl)aminomethane. The final concentration of nitrotrypsinogen was 7·3 mg per ml of the

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mixture which was 0·05 or 0·5M in $CaCl_2$. The mixture was temperature-controlled at 0°C and the activation was effected by the addition of 1·5% of trypsin with respect to the weight of nitrotrypsinogen. The activity of the activation mixture was checked by both methods for 6 days. In experiments with the digestion of the B-chain of insulin, the solution activated for 48 h was used either as such or after the evaporation to dryness.

The N-*terminal end groups* of nitro derivatives of trypsin and trypsinogen, respectively, were determined by dinitrophenylation in 8M urea[4], dissolved in 0·1M-Tris-HCl buffer at pH 8·0, containing 0·05M-$CaCl_2$ and 0·05M-NaCl. *Digestion of* B-*chain of insulin.* The following conditions of digestion by active trypsin were found to be optimum: enzyme to substrate ratio 1 : 4 000, pH 8·5, temperature 25°C. The digestion was discontinued after 30 min by acidification by acetic acid to pH 4. The digestion products were identified by the technique of peptide maps on paper (*1st* direction, electrophoresis at pH 1·9, 3 000 V (ref.[4]), *2nd* direction, chromatography in the system n-butanol–acetic acid–pyridine–water[5]). The products of digestion by nitrotrypsin were identified by the same technique. *The preparative-scale digestion* by nitrotrypsin (prepared by activation of nitrotrypsinogen) was carried out with 11·6 mg (4 μmol) of the B-chain of insulin dissolved in 8 ml of water. The pH of the solution was adjusted to 8·5 by 0·1M solution of ammonium carbonate and 24 μl of a solution containing 0·01 μmol of nitrotrypsin was added (enzyme to substrate ratio 1 : 400). The mixture was incubated 30 min at 25°C, acidified by 5% acetic acid, and taken to dryness.

The digestion products were resolved first by preparative chromatography on 1 sheet of Whatman No 3 paper in the solvent system described above into 3 main fragments. One of the latter was further fractionated by high-voltage electrophoresis, the remaining two fragments were homogeneous. The composition of these fragments was determined by amino acid analysis. A more profound cleavage of the B-chain of insulin was achieved by digestion at an enzyme to substrate ratio of 1 : 100 for 4 h at 37°C. The digestion products were identified by the technique of peptide maps as described above.

RESULTS AND DISCUSSION

The activation of nitrotrypsinogen proceeds very slowly[2]. Its time profile, as determined by the measurement of activity using both BAEE and TAME as substrates in the presence or absence of calcium, is shown in Fig. 1. This profile clearly shows that autoactivation does not proceed and that the effect of calcium is similar to that observed in experiments with the activation of trypsinogen.

In an effort to establish whether the activation of nitrotrypsinogen involves the cleavage of its peptide chain at the usual site, *i.e.* of the bond between residues Lys^6-Ile^7, the N-terminal end groups of nitrated trypsin derivatives were determined. It was found, however, that nitrotrypsinogen did not react with dinitrofluorobenzene at all while nitrotrypsin did react and the presence of DNP-valine and DNP-isoleucine was detected. This leads us to assume that the N-terminal valine of nitrotrypsinogen in polymerous state is masked inside the polymer and that the bond Lys^6- Ile^7 is accessible to tryptic attack only with great difficulties. Hence, in the process of nitrotrypsinogen activation by trypsin the bond Lys^6-Ile^7 is cleaved and the low rate of this reaction is most likely due to the lower accessibility of this bond. The function of calcium remains unaffected by nitration. To polymerization can be

ascribed most likely also the inability of nitrotrypsin to undergo autoactivation as well as the earlier reported[3] failure of nitrotrypsin to cleave high molecular weight substrates.

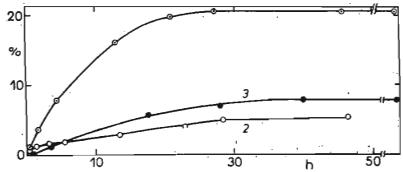

FIG. 1

Course of Activation of Nitrotrypsinogen Expressed in Per Cent of Original Potential Activity of Trypsinogen

1 Determined with BAEE as substrate in 0·05M and 0·5M-$CaCl_2$, *2* the same test in the absence of $CaCl_2$, *3* determined with TAME as substrate in 0·05M-$CaCl_2$, h time of activation.

For these reasons we wanted to determine whether there is a difference in the specificity of cleavage by nitrotrypsin and trypsin. We used as substrate the B-chain of insulin in which the disulfide bonds had been cleaved by S-sulfonation in order for tyrosine to remain possibly intact. The peptide map of the B-chain of insulin digested by nitrotrypsin which had been prepared by the activation of nitrotrypsinogen gave the same pattern as the digest prepared with active trypsin or with trypsin prepared by the nitration of active trypsin. The arising fragments were isolated only in the first case and identified by the determination of their amino acid composition which corresponded to fragments of the B-chain of insulin (Fig. 2). As obvious from the scheme, nitrotrypsin (prepared both by the activation of nitrotrypsinogen and by the nitration of trypsin) shows the same specificity of cleavage as active trypsin. In the case of nitrotrypsin prepared from nitrotrypsinogen, however, a larger amount of the enzyme had to be used for the cleavage, in proportion to ist lower total activity. We have also essayed a more profound cleavage of the B-chain of insulin and observed the additional nonspecific activity of the trypsin preparation. The peptide map pattern was again the same in all three cases and when compared with the reported cleavage[6] it corresponded to chymotryptic cleavage.

The results of the experiments described above show that nitrotrypsin (regardless of the method of its preparation) retains the activity of trypsin. Neither is affected by nitrations its nonspecific chymotryptic activity. This observation is in agreement with the data reported by French authors[3] who assume that nitrotrypsin retains full tryptic activity and that a decrease, if any, in its activity can be explained by denaturation, *i.e.* that in such a case the enzyme represents a mixture of fully active and entirely inactive molecules. According to these authors this holds true for low molecular weight

substrates such as BAEE and BANA, but not for high-molecular weight substrates, such as chymotrypsinogen, casein, or hemoglobin. It would appear, through, that it is rather difficult to draw a line dividing high molecular weight and low molecular weight substrates since insulin must necessarily by regarded as a high molecular weight substrate.

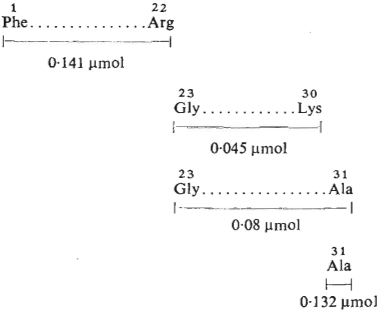

FIG. 2

Scheme of Cleavage of B-Chain of Insulin by Nitrotrypsin

The numerals designate the position of each amino acid residue in the B-chain, μmol represent the yields of individual fragments after preparative chromatography.

To explain all these properties it would be probably necessary to compare data obtained exclusively with unpolymerous, homogeneous, unambiguously defined derivatives of trypsin. Since trypsin itself is heterogeneous[7], a fact which has not been considered in the studies reported so far, we must regard all the data obtained as yet as preliminary and requiring corroboration in more detailed studies on homogeneous components of trypsin.

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