THE REACTION OF TYROSYL RESIDUES OF BOVINE TRYPSIN AND TRYPSINOGEN WITH TETRANITROMETHANE

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Several studies have shown that there are two sets of tyrosyl residues in bovine trypsin and trypsinogen (Inada et al., 1963; Smillie and Kay, 1964; DeLaage and Lazdunski, 1965; Riordan et al., 1965; Hachimori et al., 1966). Of the ten residues of tyrosine in trypsin or trypsinogen, one set of five or six is "exposed", probably occupying surface positions, and the remaining five or four are "buried". Tetranitromethane (TNM) has been recently introduced as a reagent which preferentially nitrates tyrosyl (and oxidizes cysteinyl) residues in proteins (Riordan et al., 1966, 1967, 1968) and its usefulness in probing the functional role of tyrosyl residues in several enzymes has been demonstrated (Sokolovsky et al., 1966). It appeared of interest to apply this reagent to trypsinogen and trypsin in order to test the selectivity of nitration of tyrosyl residues and the effects of the resulting modification on the activation of the zymogen and the activity of the enzyme.

Experimental

Trypsin (lot TR 7JA) and trypsinogen (lot TG 6423) were purchased from Worthington Biochemical Corporation. Tetranitromethane was obtained from Aldrich Chemical Company, and 3-nitrotyrosine from K&K Laboratories. p-Nitrophenyl-p'-guanidinobenzoate HCl (NPGB) was synthesized and used according to Chase and Shaw (1967).

Nitration with TNM was carried out at 23°, pH 8.0 (0.05 M Tris-HCl), 0.05 M $CaCl_2$, 5% ethanol, and 10 mg protein per ml. Nitration of tyrosyl residues was quantitated by absorbance at 355 mμ at pH 3 (Riordan et al., 1967). Nitrated proteins were reduced and S-aminoethylated with ethylenimine (Raftery and Cole, 1963) prior to digestion with α-chymotrypsin. The digests were desalted and fractionated on Sephadex G-15 or G-25 using 5% acetic acid as an eluent. The isolated peptides were subsequently purified on Dowex 1X2 or SE-Sephadex C-25, using gradients of pyridine-acetate to develop the columns. Final purification was achieved after fractionation on Dowex or SE-Sephadex by high voltage paper electrophoresis.

3-Nitrotyrosine was identified in acid hydrolysates of nitrated proteins on a Spinco Model 120 amino acid analyzer (Sokolovsky et al., 1966) in 80-90% yield (based on the ninhydrin color value for tyrosine). Based on absorbance at 355 mμ, similar yields of nitrotyrosine were observed for acid hydrolysates of peptides containing nitrotyrosine. Amino-terminal analyses of the peptides were performed by a subtractive Edman method (Konigsberg and Hill, 1962).

Trypsin, nitro-trypsin, and nitro-trypsinogen were chromatographed on SE-Sephadex C-50 at pH 3.1 using a modification of the method developed by M. Sanders in this laboratory (unpublished) (Fig. 1A). In either case, nitration had no effect on the chromatography at pH 3.1. Peak I was further purified by separation from inert material on SE-Sephadex using 0.35 M NaCl (Fig. 1B). Nitro-trypsinogen was freed from inert protein using the same column as in Fig. 1B.

Results and Discussion

The time courses of reaction of trypsin and trypsinogen with a 50-fold molar excess of TNM over tyrosine are given in Fig. 2. The rate and extent of nitration were similar for the enzyme and the zymogen. Assuming that maximally six tyrosyl residues were nitrated, a plot of the data according

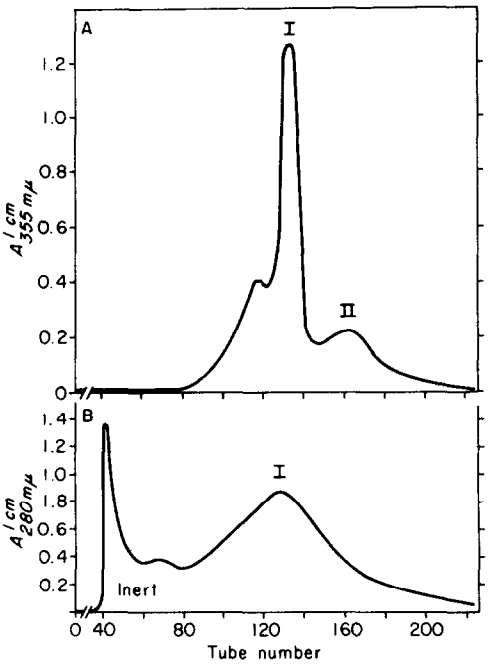

Fig. 1: Chromatography of Nitro-Trypsin. A: Chromatography of nitrotrypsin on a column of SE-Sephadex C-50 (2.5X110 cm). Nitrotrypsin was eluted with 0.5 M NaCl buffered at pH 3.1 with 0.005 M Na-citrate at 4°. The flow rate was 20 ml/hr, and 4.2 ml fractions were collected. B: Rechromatography of peak 1 on SE-Sephadex C-50 using a column 2.5X40 cm and eluted at pH 3.1 with 0.35 M NaCl. All other conditions were identical to A.

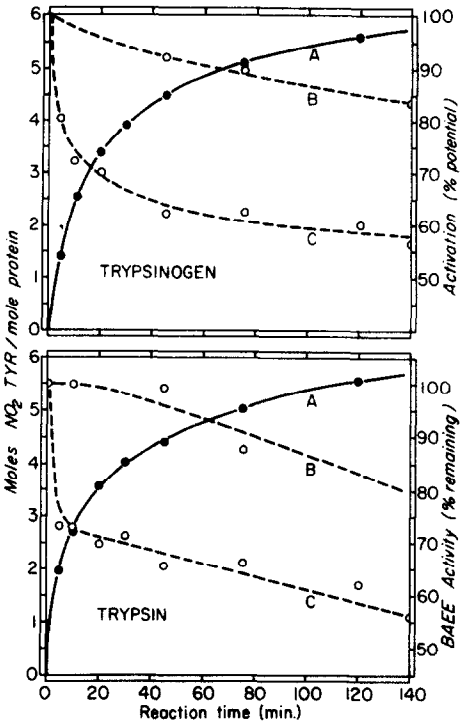

Fig. 2: Time-Course of TNM Reaction with Trypsin and Trypsinogen. A 50-fold molar excess of TNM to tyrosine was used under conditions described in the text. The upper half of the figure is the reaction of TNM with trypsinogen and the lower half with trypsin. Curve A is the number of nitrotyrosyl residues formed with time (spectrally determined), curve B is the activity or activatability for controls minus only TNM, and curve C represents the change in activity or activatability of the nitrated protein.

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to Koshland et al. (1958) as log % unmodified tyrosine against time of nitration revealed that between two and three tyrosyl residues were more rapidly nitrated than the remainder, in both the enzyme and the zymogen. The value of five to six reactive tyrosyl residues obtained in several experiments agrees well with other methods of probing the reactivity of tyrosyl residues (Inada et al., 1964; DeLaage and Lazdunski, 1965; Riordan et al., 1965; Hachimori et al., 1966).

Concomitant with nitration (using a 50-fold molar excess of TNM with respect to tyrosine), there was a partial loss in activity of trypsin (ca 25%) and activatability of trypsinogen (35%) over the native control, corresponding in each case to the nitration of approximately three tyrosyl residues (Fig. 2). There was no substantial loss in either property upon more extensive nitration. Fig. 3 illustrates that the level of nitration of trypsin could be controlled by varying the ratio of TNM to trypsin using 60 min. reaction times throughout. The introduction of two residues was accompanied by an apparent loss of 25% of the esterase activity. Reaction conditions were adjusted so as to yield preparations containing 1.0 and 1.8 nitrotyrosine residues per protein molecule. Chromatographic separation from inert protein by the procedure described above yielded in each case a nitrated enzyme that was fully active against BAEE.

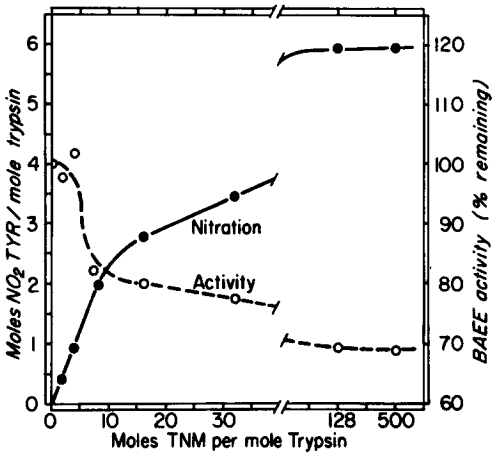

Fig. 3. Titration of Trypsin with Low Molar Excess of TNM. The reaction was stopped in each case at 60 min. by addition of sufficient 1 N HCl to decrease the pH to 3. For details of the conditions used, see the text.

Trypsinogen was similarly reacted with a four-fold molar excess of TNM for 60 minutes and found to contain 1.0 nitro group per protein molecule. Following chromatographic purification, the maximum extent of activation as judged by activity toward BAEE was 80% of the unmodified control (Fig. 4). Active site titrations with NPGB after activation for 150 minutes indicated 0.91 active sites per molecule as compared to 0.87 for the unmodified control.

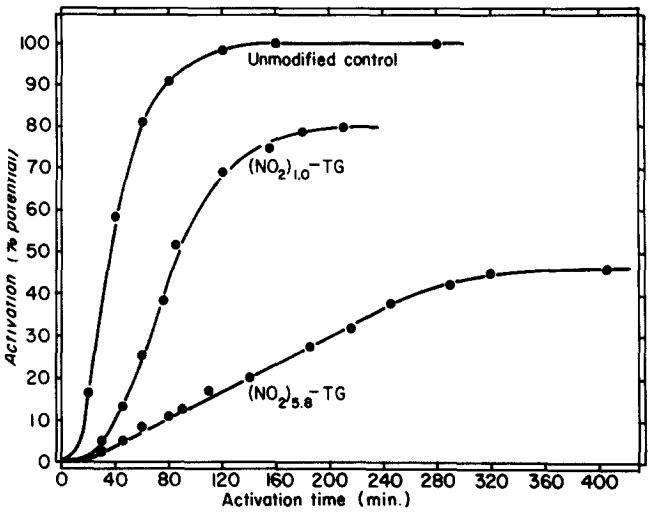

Fig. 4. Activation of Nitrated Trypsinogen. "Lightly nitrated" trypsinogen was modified with 1.1 nitrotyrosyl groups per molecule, and "heavily nitrated" was fully nitrated with 5.8 groups. Activation was accomplished by addition of 1:15 trypsin at pH 8.3 (0.1 M tris-HCl, 0.05 M $CaCl_2$), 0°. The zymogen concentration was approximately 5 mg/ml in each case. Activity generated was determined against 10^{-2} M BAEE at pH 7.8 in a pH stat.

Chymotryptic digestion of trypsin containing 1.0 nitrotyrosyl group per molecule yielded four nitrotyrosyl peptides, representing tyrosyl residues 137, 11 and 28 (Table I). In a similar experiment, the nitrotyrosine-containing peptides were isolated and identified from trypsin containing 1.8 nitrotyrosyl groups per molecule (Table I). Additional TNM over that necessary to nitrate one tyrosyl per molecule appeared only to increase the amount of nitration of the three tyrosy residues and to nitrate a fourth group, tyrosine 48. Isolation of nitrotyrosyl peptides from nitrotrypsinogen containing 1.4 nitro groups per molecule followed the pattern described for trypsin.

Nitrotyrosyl peptides were also isolated from heavily nitrated proteins (5.8 nitrotyrosyl groups per molecule). From both the enzyme and the zymogen, peptides containing nitrotyrosyl residues 11, 28, 48 and 137 were obtained. In addition, a peptide containing nitrotyrosyl residue 171 was found, corresponding to SAEcys^{168}-ala-gly-NO_2tyr^{171}.

There is one interesting difference in the peptide patterns of the enzyme and zymogen. More than 85% of the isolated peptides containing nitrotyrosyl residue 137 from trypsin were derived from a split between lysine^{131} and serine^{132}. In trypsinogen, the peptide containing nitrotyrosyl residue 137 was derived from a split between threonine^{135} and serine^{136}. It is probable

Table I

SUMMARY OF YIELDS OF NITROTYROSINE PEPTIDES ISOLATED FROM NITRATED TRYPSIN AND TRYPSINOGEN

	$\frac{\mu mole\ Peptide\ Recovered}{\mu mole\ Protein}$	% of NO_2tyr Recovered
Trypsin modified with 1.0 nitrotyrosyl residues		45
ser^{132}-ser-gly-thr-ser-NO_2tyr^{137}-pro-asp-val-leu^{141}	0.27	
ile^{7}-val-gly-gly-NO_2tyr^{11}	0.06	
ser^{26}-gly-NO_2tyr^{28}	0.08	
ser^{136}-NO_2tyr^{137}-pro-asp-val-leu^{141}	0.04	
Trypsin modified with 1.8 nitrotyrosyl residues		57
ser^{132}-ser-gly-thr-ser-NO_2tyr^{137}-pro-asp-val-leu^{141}	0.32	
$SAEcys^{47}$-NO_2tyr^{48}	0.15	
ile^{7}-val-gly-gly-NO_2tyr^{11}	0.18	
ser^{26}-gly-NO_2tyr^{28}	0.09	
asn^{25}-ser-gly-NO_2tyr^{28}	0.13	
ser^{136}-NO_2tyr^{137}-pro-asp-val-leu^{141}	0.17	
Trypsinogen modified with 1.4 nitrotyrosyl residues		47
ser^{136}-NO_2tyr^{137}-pro-asp-val-leu^{141}	0.33	
ser^{132}-ser-gly-thr-ser-NO_2tyr^{137}-pro-asp-val-leu^{141}	trace	
ile^{7}-val-gly-gly-NO_2tyr^{11}	0.19	
ser^{26}-gly-NO_2tyr^{28}	0.19	

that nitrotrypsin isolated by our method was principally in the "α" form with an intrachain split between lysine^{131} and serine^{132} as described by Schroeder and Shaw (1968).

The evidence presented in this preliminary communication indicates six tyrosyl residues can be maximally nitrated on native trypsin and trypsinogen. These, presumably, are the same "surface residues" that have been discerned by other methods, such as acetylation or spectrophotometric titrations (Riordan et al., 1965; DeLaage and Lazdunski, 1965). Of these six tyrosyl residues, three to four are more readily nitrated in the presence of relatively low molar excesses of TNM than the remainder. Two residues, in particular, show preferential kinetic reactivity. Of the four tyrosyl residues that have been identified by peptide isolation from chymotryptic digests of lightly-nitrated trypsin and trypsinogen, i.e. residues 137, 11, 28 and 48, none appears so reactive as to be modified at the exclusion of the others. Recovery data suggest, however, that in all cases, tyrosine 137 may have been more extensively nitrated than the remainder. Except for small changes in rate constants, nitration of up to four tyrosyl residues has no effect on the activity of trypsin or on the activatability of trypsinogen.

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