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POSITION OF REACTIVE TYROSINE RESIDUES IN TRYPSINOGEN

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By the nitration of trypsinogen by tetranitromethane, a preparation containing 5·4 residues of nitrotyrosine in the molecule was obtained. After the activation by trypsin, which was very slow, the nitrated enzyme showed considerably lower esterase activity than the parent trypsin. After the disruption of disulfide bonds and enzymatic hydrolysis of the modified protein, all peptides containing either tyrosine or nitrotyrosine were isolated. The obtained results were compared with our earlier data on trypsin and differences were found. Evidence was obtained that the total number of nitrated tyrosine residues is the same for both proteins. After the activation, however, residues No 20 and 28 show increased reactivity while residues No 82 and 171, and to a certain degree also residue No 48, on the contrary decreased reactivity as compared with the values found for trypsinogen.

In our earlier study[1] on the topography of trypsin we have shown that the nitration of tyrosine residues by tetranitromethane[2,3] with the subsequent isolation of all peptides containing tyrosine or nitrotyrosine is an elaborate yet useful method furnishing sufficient information permitting the reactivity of each of the 10 tyrosine residues of trypsin to tetranitromethane to be characterized at least semiquantitatively. Even in our preliminary experiments[4,5] we found certain differences in the reactivity (and thus in sterical accessibility) of certain residues in trypsinogen and in trypsin. These differences may throw light on topographical changes which occur in the process of trypsinogen to trypsin activation. Similarly, the activity of nitrotrypsin formed by the activation of nitrotrypsinogen considerably differs from the activity of trypsin nitrated in the form of enzyme.

For these reasons we performed an analogous study on the nitration of trypsinogen and isolated all peptides containing tyrosine or nitrotyrosine. Simultaneously with our previous paper[1] an analogous report of American authors[6] appeared. This study,

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with which our results are in good agreement, dit not supply, however, enough data permitting the characterization of differences in the reactivity of tyrosine residues in the two proteins.

EXPERIMENTAL

Material and Methods

Trypsinogen, *trypsin*, and *tetranitromethane* were characterized in the preceding paper[1].

Nitration. The procedure described earlier[1-3] was employed except that 0·5% (by weight) of soy-bean trypsin inhibitor was added. Trypsinogen (750 mg) was dissolved in 75 ml of Tris-HCl buffer (0·1M + 0·05M-KCl + 0·05M-$CaCl_2$) at pH 8·0, which contained 3·75 mg of soy-bean trypsin inhibitor. This solution was nitrated by the addition of 1·42 ml of the tetranitromethane solution (*i.e.* an approximately 4-fold molar excess of the reagent per total number of tyrosine residues of trypsinogen). The reaction mixture was stirred at room temperature and after 90 minutes the reaction was discontinued by acidification with acetic acid to pH 3·5. Nitrotrypsinogen was isolated by passage of the reaction mixture over a Sephadex G-25 column equilibrated with 0·25% acetic acid.

Determination of concentration of solutions of trypsin, trypsinogen, and their nitro derivatives was carried out by measurement of the absorbance of the solutions at 280 nm and pH 3·0. For the determination of the extinction coefficient, nitrotrypsinogen (an equivalent taken from the main peak obtained by gel filtration of the reaction mixture over Sephadex G-25) was taken to dryness and the dry residue (16·1 mg) dissolved in 1·61 ml of the buffer used for activation. A 50 µl aliquot of this solution was pipetted into 3 ml of 0·001N-HCl and the absorbance at 280nm of the resulting solution was measured. The extinction coefficient in 0·1N-NaOH was determined with the same solution at the maximum absorption at 283 nm. The values of the extinction coefficients are given in Table I.

Determination of tryptic activity in the nitration mixture or after the isolation on Sephadex was performed spectrophotometrically with tosyl-arginine methyl ester (TAME)[7] or by the titration procedure using benzoyl-arginine ethyl ester (BAEE).

TABLE I

Extinction Coefficients of 1% Solutions of Proteins

Enzyme	$E^{1\%}_{280}$
Trypsin	15·4[a]
Trypsinogen twice crystallized	14·9[b]
Nitrotrypsinogen at pH 3·0[c]	19·2
Nitrotrypsinogen in 0·1M-NaOH[d]	$E^{1\%}_{283} = 20·3$

[a] *cf.* ref.[10]; [b] The value was derived from that for twice crystallized trypsin with respect to the ratio of molecular weights.; [c] *cf.* Experimental; [d] The maximum extinction in 0·1M-NaOH lies at 283 nm.

TABLE II

Tyrosine and Nitrotyrosine Peptides Isolated from Nitrotrypsinogen and from Nitrotrypsin

Designation	Composition[a]	Tyr No[a]	Quantity[b], μmol		Relative content, %			
			isolated	original	Tyr	NO_2Tyr	Tyr	NO_2Tyr
					in nitrotrypsinogen		in nitrotrypsin[c]	
55B	^{6}Lys(Ile,Val,Gly,Gly,NO_2Tyr^{11})	11	2·0	24	—	100	—	100
10A	^{12}Thr(Cys,Gly,Ala,Asn,Thr,Val,Pro,Tyr^{20})	20	0·60	1·2	80	20	40	60
10 B2	^{12}(Thr,Cys,Gly,Ala,Asn,Thr,Val,Pro,Tyr^{20})		0·30	1·2				
24 AB′2	^{12}(Thr,Cys,Gly,Ala,Asn,Thr,Val,Pro,Tyr^{20}) (55%)[d] ^{12}(Thr,Cys,Gly,Ala,Asn,Thr,Val,Pro,NO_2Tyr^{20}) (45%)[d]		0·12	0·96				
30 BC′2	^{25}(Asn,Ser,Gly,Tyr^{28})	28	0·03	1·24	28	—	—	
29 B′1	^{25}(Asn,Ser,Gly,NO_2Tyr^{28})		0·156	0·62		72		100
31 C	^{41}(Val,Val,Ser,Ala,Ala,His,Cys,NO_2Tyr^{48})	48	0·15	2·1	—	100	10	90
41 BD′2	^{41}(Val,Val,Ser,Ala,Ala,His,Cys,NO_2Tyr,Lys^{49})		0·25	1·0				
44 B	^{76}(Ser,Ile,Val,His,Pro,Ser,NO_2Tyr^{82})	82	0·035	0·42	53	47	75	25
45 CC′	^{76}(Ser,Ile,Val,His,Pro,Ser,NO_2Tyr^{82}) (60%)[d]		0·140	0·560	—			
	^{76}(Ser,Ile,Val,His,Pro,Ser, Tyr^{82}) (40%)[d]							

25 Cl	^{132}(Ser,Ser,Gly,Thr,Ser,NO_2Tyr)^{137}	137	0·35	2·15	—	100	—	100
22 C′1	^{156}Ser(Ala,Tyr,Pro,Gly,Gln,Ile,Thr,Ser,Asn)^{165}	158	0·81	2·73	100	—	100	—
25 H	^{155}(Lys,Ser,Ala,Tyr,Pro,Gly,Gln,Ile,Thr,Ser,Asn,Met)^{166}		0·187	0·75				
16 A	^{168}(Cys,Ala,Gly,NO_2Tyr)^{171}	171	0·55	6·6	—	100	38	62
23 A	^{168}(Cys,Ala,Gly,NO_2Tyr,Leu)^{172}		0·052	0·16				
16 A″2	(Ala,Gly,NO_2Tyr)		0·178	0·712				
40 D1	^{207}Asn(Lys,Pro,Gly,Val,Tyr)^{212}	212	0·39	10·8	100	—	100	—
40 C1	(Asp,Lys,Pro,Gly,Val,Tyr)		0·172	0·7				
32 E′2	^{213}Thr(Lys,Val,Cys,Asp,Tyr)^{218}	218	0·17	0·68	100	—	100	—
18 B′	Val(Cys,Asn,Tyr)		0·9	5·4				
		Total			461	539	463	537

[a] Serial number of amino acid residues in the structure of trypsinogen[11,12] (*cf.* Fig. 1). [b] The isolated quantity of the peptide was calculated from its amino acid analysis. The quantity of the peptide originally present in the digest was estimated on the assumption of 50% losses during the fractionation by paper techniques (paper chromatography and electrophoresis). [c] Analogous results for trypsin described in our preceding paper. [d] Relative tyrosine and nitrotyrosine content of peptide according to amino acid analysis.

Activation of nitrated trypsinogen was effected at 0°C in 0·02M Tris-HCl, at pH 8·0, containing 0·05M-$CaCl_2$, by the addition of 1·5% of trypsin. The concentration of nitrotrypsinogen was 7·5 mg/ml. The aliquots were withdrawn at various intervals over a period of 75 hours.

The number of nitrated tyrosine residues was determined in the solution obtained by gel filtration of the reaction mixture by the procedure described in our previous paper[1].

S-*Sulfonation* was carried out with a total amount of 880 mg (36 μmol) of nitrotrypsinogen (total volume 65 ml after concentration) following essentially the procedure described by Pechère and coworkers[8]. S-Sulfo-nitrotrypsinogen was isolated from the reaction mixture by gel filtration on a column of Sephadex G-25 equilibrated with 0·2M-$(NH_4)_2CO_3$. The effluent was taken to dryness in a rotary evaporator at 40°C.

Combined enzymatic hydrolysis. The dry residue was suspended in water and dissolved by the addition of 0·1M-NaOH to pH 8·5. The total volume of the solution was 50 ml. The opalescent solution was temperature-controlled at 37°C and the enzymes (1% by wt.) were added in the order trypsin, chymotrypsin, again trypsin, and pepsin. The pH was checked at short intervals by a glass electrode and maintained at the value of 8·5 by the addition of 0·1M-NaOH. With regard to the alkali uptake the tryptic digestion was discontinued after 13 hours, the chymotryptic digestion after 19 hours. After the second addition of trypsin, the alkali uptake was negligible. Three hours later the reaction mixture was acidified by the addition of 1M-HCl to pH 2. At approximately pH 3 a precipitate had formed. Pepsin was added to the suspension and the pH was maintained at 1·95 by the addition of 1M-HCl for 35 hours. The insoluble portion of the digest was centrifuged off, washed twice with 0·001M-HCl, lyophilized, weighed and subjected to amino acid analysis. The supernatant and the washings were pooled and also subjected to amino acid analysis.

Fractionation of peptides was performed by ion-exchange chromatography and by paper techniques as described in our preceding paper[1]. The peptides were identified by quantitative amino acid analysis and by N-terminal end-group analysis by the dansyl technique[9].

RESULTS

Nitration and gel filtration. The yield of these operations was 91% (in terms of protein content). The esterase activity of trypsinogen was zero both before and after the nitration. The activation of nitrotrypsinogen by 1·5% of trypsin proceeded very slowly (40 hours) and with various results for the same sample. The activity of the product toward BAEE was 21%, toward TAME only 8% of the original potential activity of unmodified trypsinogen. The tyrosine content of the nitrotrypsin solution after gel filtration determined spectrophotometrically was 4·7 residue, of the solution after enzymatic hydrolysis 4·6 residues (determined by amino acid analysis). The nitrotyrosine content of these solutions was lower than 5·3 residues, a value which would correspond to a difference between 10 and 4·7 residues.

As obvious from Table II, the sum of the relative tyrosine content of all peptides was 461%, and of the relative nitrotyrosine content 539%. Thus, according to the amino acid analyses of all the isolated peptides, altogether 5·4 residues were nitrated and 4·6 residues remained intact. This data confirm our earlier finding[1] that the analytical values provide reliable information merely on the number of unmodified tyrosine residues whereas nitrated tyrosine residues obviously undergo additional changes

and escape analysis both chemical (do not react with ninhydrin) or spectrophotometrical.

The enzymatic digest was practically all soluble since the weight of the insoluble portion was only 2 mg (*i.e.* 0·23% of the starting material). This material contained only traces of tyrosine and nitrotyrosine and was therefore not considered. By contrast, the soluble portion corresponded according to amino acid analysis practically to the whole molecule of trypsinogen. The tyrosine value was 4·6 residues. Therefore the soluble portion of the digest from which the peptides were isolated represented whole nitrotrypsinogen.

All peptides containing tyrosine or nitrotyrosine were isolated and are summarized in Table II. From the isolated quantity of each individual peptide its content in the parent hydrolysate was estimated. From these values the relative ratio of nitrated to intact tyrosine was derived for all ten tyrosine residues of trypsinogen. These

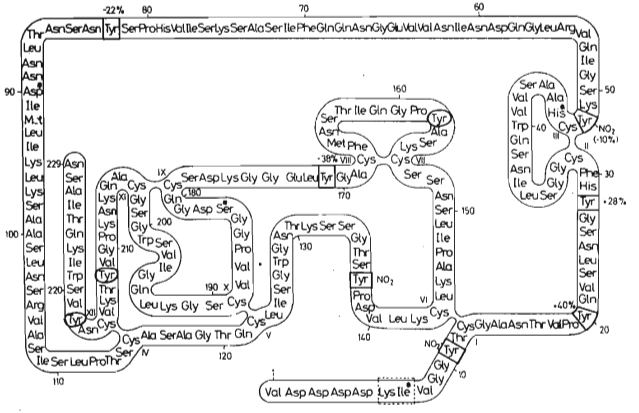

FIG. 1

Schematic Representation of the Structure of Trypsinogen[12] with the Marked Positions of Tyrosine Residues

Tyrosine residues nitrated completely in both trypsinogen and trypsin are marked by the symbol —NO_2. The remaining tyrosine residues are either encircled (those which are not nitrated at all) or marked by the value in per cent which indicates whether they are nitrated to a higher degree (+) or to a lower degree (−) in trypsin than in trypsinogen. The bond Lys^6–Ile^7 cleaved with difficulties after the nitration of trypsinogen is marked by a dashed line.

results are put side by side with similar data on trypsin[1] in Table II from which the changes in the reactivity of individual tyrosine residues caused by the activation of trypsinogen to trypsin are obvious. These changes are shown in the schematic structure of trypsinogen[12] (Fig. 1) and are expressed in per cent of reactivity or rather "unmasking" (+) or "masking" (−) of individual residues. These results provide further and more exact evidence in favor of our previous findings concerning the neighborhood of residue No 82. At the same time the total of nitrated and intact tyrosine residues is practically the same in both cases, *i.e.* 5·4 nitrated residues and 4·6 intact residues (Table II). This value is in perfect agreement with the analytical value for tyrosine residues remaining intact after the nitration and also with the data of other authors[13–16] on the 5–6 reactive tyrosine residues.

DISCUSSION

This paper had been already in an advanced stage when the report appeared by Shaw and coworkers[17,18] describing the resolution of trypsin into three partially cleaved components α, β, and ψ. After our study had been completed, reports on the formation of polymers during nitration appeared[19,20]. These studies show that our results cannot be interpreted directly in terms of changes in the topography of the molecule during trypsinogen to trypsin activation, since the changes observed by us could be influenced also by the effects of polymeration. Moreover, trypsin which had been regarded as homogeneous, is a mixture of at least three kinds of molecules which may or may not differ in three-dimensional structure.

All the papers on the structure of trypsin which have been published as yet (save for Shaw's[17,18] papers) present results which reflect average properties of all three kinds of trypsinogen.

In view of the fact that we have isolated all peptides containing tyrosine, either nitrated or intact, our results are unambiguous (*i.e.* not affected by polymeration) in those cases where 100% of the intact form was found, that is in the case of three residues, No 158, No 212, and No 218. Vincent and coworkers[19] found in the purified monomer of nitrotrypsinogen 5 tyrosine residues and 5 nitrotyrosine residues. It follows, however, from a comparison of this data with our results that the found number of 5 unmodified tyrosines represents merely 3 residues actually unmodified while the remaining 2 residues an average value, characterizing several residues substituted only partly. We cannot assume namely that during the polymeration additional, until then unaccessible tyrosine residues could react. On the contrary, the number of 5 nitrated residues in the monomer is in agreement both with our average value of 5·4 (*cf.* sum in Table II) and also with the data of American authors[6] who identified only 5 nitrated residues (No 11, 28, 48, 137, and 171). These authors also carried out the reaction under conditions not preventing polymeration. Since they isolated only peptides containing nitrated tyrosine, the peptides with low nitro-

tyrosine content (No 20 and 82) obviously escaped their analyses even in the case of completely nitrated derivatives.

Since these authors were unable to balance the numbers of all residues, they could not observe the differences in the degree of nitration of trypsinogen and trypsin found by us, because these differences between average values for the two proteins become zero (*cf.* total sums in Table II).

The existence of certain tyrosine residues in both forms could be the result of polymeration. Even in those cases, however, we find a remarkable agreement with the data of Shaw[17] and an analogy to chymotrypsin[21]. Thus, *e.g.* residue No 82, which we have observed as "masked" during activation (*cf.* Table II), occupies a position analogous to tyrosine No 94 in chymotrypsinogen which shows decreased reactivity[22] and participates on the hydrophobic envelope of aspartic acid No 102 (in trypsinogen No 90). This aspartic acid residue participates on the enzymatic mechanism by a hydrogen bond linking histidine No 57 (in trypsinogen No 46) to serine No 195 (in trypsinogen No 183) (ref.[23]).

Another residue which we found "masked" after the activation is tyrosine No 171 situated in the neighborhood of the binding site for the substrate (Asp 177) according to the information provided by both the primary structure and a three-dimensional model[21]. We found "unmasked" two other tyrosine residues, No 20 and 18, which are nitrated partly in the shielded area and partly on the surface of the molecule.

The completely nitrated residues No 11, 48, and 137 are situated, also according to the three-dimensional model, exclusively on the surface of the molecule, while intact residues No 158, 212, and 218 are, on the contrary, in the hydrophobic area. Residue No 158 would appear, at a first glance, to be distinctly different from residue No 171 in chymotrypsin, which has been found fully reactive[22]. Tyrosine No 158, however, is four residues distant from disulfide bond VII–VIII and thus situated in the position of tryptophan (chymotrypsinogen No 172), which is turned toward the hydrophobic core of the molecule[24]. This difference in the reactivity of the two tyrosines (trypsinogen No 158 and chymotrypsinogen No 171) provides another piece of evidence of the close analogy in the three-dimensional structure of both enzymes.

Our data is also in accordance with the results obtained recently with the modification of tyrosines by other reagents: Gorbunoff[25] found in trypsinogen 5–7 reactive residues of different degree of reactivity, *i.e.* in the order of decreasing reactivity, 3 + 1 + 1 residues when he labeled the enzyme with acetylimidazole, and 2 + 1 + + 1 + 1 + 2 residues when using cyanuric fluoride. These values are in good agreement with our data when they are arranged in the order of decreasing relative content of nitrotyrosine (*cf.* Table II), *i.e.* No 11, 137, 48, 171, 28, 82, and 20.

It could be objected that the partly substituted residues might represent a certain amount of the denatured protein with impaired three-dimensional structure. This, however, would be in disagreement with the observation that certain residues in tryp-

sin show decreased reactivity as compared to the same residues in trypsinogen, while others show on the contrary increased reactivity.

The composition of the isolated peptides, also of those which do not contain tyrosine, shows that the susceptibility of the peptide chain of trypsinogen to cleavage by trypsin remained practically unaltered by the nitration, save for one bond, which, however, is of fundamental importance. This is the bond Lys^6-Ile^7, which is essential for the formation of active trypsin. The fact that this bond is cleaved with difficulties is evidenced by the existence of basic peptide 55B (Table II), which was isolated in the highest yield of all peptides (almost 70%) and as the only one from this region. The difficulties with which the cleavage proceeds are indicated also by the abnormally long period required for the activation of nitrotrypsinogen (40 hours).

The American authors[6] found instead of our peptide 55B a peptide shorter by one lysine residue at its N-terminus, *i.e.* Ile^7—NO_2Tyr^{11}. This finding is in disagreement with our results and also with the assumption of chymotryptic cleavage by which these authors explain their results. The other peptides isolated by these authors are in agreement with our data. It is possible that the inacessibility of this bond to tryptic cleavage is caused by polymeration during nitration and that the low activity of the enzyme formed by the activation of nitrotrypsinogen corresponds only to a low amount of the monomer. Since we have observed after the activation of nitrotrypsinogen certain differences in the activity of the formed enzyme toward TAME and BAEE and since we did not observe autoactivation – a finding which is in agreement with the data of French authors[19] – it seems logical to investigate these problems in one of our future studies.

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