NITRATION BY TETRANITROMETHANE OF THE COMPLEX OF CHYMOTRYPSIN WITH THE BASIC PANCREATIC TRYPSIN INHIBITOR

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The tyrosine residues of chymotrypsin are equally reactive towards tetranitromethane in the free enzyme and in a complex with the trypsin inhibitor. The role of a tryptophan residue of the enzyme in this interaction is thus emphasised.

In previous work[1], the behaviour of the basic pancreatic trypsin inhibitor during its interaction with chymotrypsin was examined. The circular dichroic difference spectrum which accompanies formation of the complex implicates either a tyrosine or a tryptophan residues in the interaction. Since nitration of the tyrosine residues of the inhibitor with tetranitromethane proceeded in the same way for both free and complex-bound inhibitor[1,2], the difference spectrum must arise from the perturbation of a tyrosine or tryptophan residue in the enzyme moiety of the complex.

In free chymotrypsin, tyrosine No 146** and No 171 react with tetranitromethane[3,4]. The purpose of the present work was to determine whether either or both of these residues becomes unreactive when chymotrypsin is bound in a complex with the trypsin inhibitor.

EXPERIMENTAL

The preparation of basic bovine pancreatic trypsin inhibitor[5] and bovine α-chymotrypsin[6] has already been described. Tetranitromethane was fractionated by discarding the part[7] which remained liquid when the temperature of the sample was lowered to 13°C.

Nitration of the proteins was carried out following Riordan and coworkers[8]. Tetranitromethane was diluted to 0·73M with 95% ethanol and added to the protein solution (0·7 mg ml^{-1}) in 0·05M Tris-HCl buffer, pH 8·0, to give a 110-fold molar excess of reagent over tyrosine residues. The reaction proceeded 60 min at pH 7·95—8·00 after which time the nitration of chymotrypsin is essentially complete[4]. The nitrated protein was separated from the reaction mixture by gel filtration on Sephadex G 25 in 0·1M-NH_4HCO_3 (pH 8·2, ref.[7]), and lyophilized.

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** The numbering system is that used for chymotrypsinogen.

The S-sulphoderivative of nitrated chymotrypsin was prepared by the method of Pechere and coworkers[9] and isolated from the reaction mixture by gel filtration on Sephadex G 25 in 0·1M-NH_4HCO_3, pH 8·2.

Chymotryptic digestion of the unlyophilized S-sulphoderivative was carried out at a molar enzyme to substrate ratio of 1 : 40 for 16 h at 37°C and pH 8·5. The digest was lyophilized.

Peptide maps were obtained with 3 mg of peptide mixture on Whatman No 3 paper. Their separation was effected by descending electrophoresis[10] at 1400 V in system S_1 (pH 5·6), acetic acid–pyridine–water (1 : 4 : 995), combined with paper chromatography in system S_2, n-butanol–acetic acid–pyridine–water (15 : 3 : 10 : 12). Nitrotyrosine-containing peptides were eluted from the paper with 0·05M Tris-HCl buffer, pH 8·0. Their relative concentrations were determined spectrophotometrically at 428 nm, the absorbance maximum[11] of nitrotyrosine under alkaline conditions.

Absorbance measurements were made on an Opton (Zeiss PMQII) spectrophotometer. For the absolute determination of nitrotyrosine content, the extinction coefficient[11] $\varepsilon = 4\,100\ M^{-1}$, pH 8, was used.

Quantitative amino-acid analyses (6M-HCl, 24 h, 110°C) were carried out by the technique of Spackman and coworkers[12] on an instrument manufactured by the Development Workshops, Czechoslovak Academy of Sciences.

Preparation of the chymotrypsin-trypsin inhibitor complex. The complex was formed by mixing a solution of 190 mg chymotrypsin in 10 ml 0·001M-HCl with 60 mg inhibitor dissolved in 5 ml water[1]. The mixture was made up to 50 ml with 0·05M Tris-HCl buffer, pH 8, and allowed to stand at this pH for 15 min. The quantities of the two proteins used correspond to 1·2 mol of inhibitor per mol of chymotrypsin; the complex was isolated from the small excess of inhibitor by chromatography on Sephadex G 75 (6·1 × 50 cm) in 0·05M-NH_4HCO_3, pH 8·2. The amino-acid analysis is given in Table I.

Dissociation of the complex. The nitrated complex, after lyophilization, was dissolved at a concentration of 5 mg ml^{-1} in 0·001M-HCl, and the solution kept at pH 3·0 for 30 min. The two components of the mixture were then separated by gel filtration on Sephadex G 75 in 0·1M-NaCl adjusted to pH 3 with 1M-HCl.

RESULTS

The amino acid composition of chymotrypsin before and after nitration is given in Table I. Significant differences are seen to occur in the number of tyrosine residues, the nitrated enzyme being to contain 1·7 mol of tyrosine and 1·0 mol of nitrotyrosine per mol of protein. Measurement of the absorbance at 428 nm, pH 8, yielded a value of 2·0 mol of nitrotyrosine per mol of nitrated chymotrypsin.

A typical peptide map derived from the chymotryptic digest of nitrated and S-sulphonated chymotrypsin is shown in Fig. 2. The tyrosine-containing peptides were designated A, B_1, B_2, C and D, in accordance with previous work[4]. Peptides A, B_1, and B_2 were isolated on a semi-preparative scale in systems S_1 and S_2, and their qualitative amino acid composition determined. From the results, peptide A was shown to contain Tyr No 146, and peptides B_1 and B_2, Tyr No 171. The earlier investigation alone allowed unambiguous identification of the peptides C and D as

TABLE I

Amino Acid Composition of Chymotrypsin, Its Complex with the Trypsin Inhibitor, and Their Nitrated Derivatives

A chymotrypsin, B nitrated chymotrypsin, C chymotrypsin fraction isolated from nitrated complex, D complex, E nitrated complex. Values for 24-hour hydrolysates, not corrected; numbers in brackets represent the amino-acid composition calculated from the primary structures of the proteins[3].

Amino acid	Number of residues in hydrolysate				
	A	B	C	D	E
Ala	22·0 (22)	22·0	22·0	28·0 (28)	28·0
Asp	21·6 (22)	22·0	21·6	25·9 (27)	25·6
Arg	2·7 (3)	2·3	2·8	8·5 (9)	8·2
Cys[a]	8·5 (10)	9·5	7·7	9·6 (16)	13·0
Glu	15·0 (15)	14·6	14·4	17·9 (18)	17·7
Gly	24·0 (23)	25·1	21·8	27·7 (29)	28·5
His	1·6 (2)	2·0	1·8	1·3 (2)	1·9
Ile	8·7 (10)	8·5	8·3	10·7 (12)	10·3
Leu	18·6 (19)	17·8	18·3	22·1 (21)	20·3
Lys	12·8 (14)	13·7	13·0	17·9 (18)	16·4
Met	2·1 (2)	1·9	2·1	3·1 (3)	3·1
Phe	5·7 (6)	5·9	5·7	10·7 (10)	10·0
Pro	7·5 (9)	9·7	6·2	15·6 (13)	13·3
Ser	24·9 (27)	26·9	25·6	25·6 (28)	25·7
Thr	21·1 (22)	21·1	21·5	23·4 (25)	23·0
Tyr	3·7 (4)	1·7	2·1	8·4 (8)	4·0
Tyr (3-NO_2)	— (—)	1·0	1·2	— (—)	2·8
Trp	[b] (8)	[b]	[b]	[b] (8)	[b]
Val	21·3 (23)	22·5	20·0	21·3 (24)	22·0

[a] Samples not oxidised before analysis. [b] Not determined.

TABLE II

Relative Amount of Nitrotyrosine in Peptides Isolated from the Chymotryptic Digest of Nitrated and S-sulphonated Chymotrypsin

The values measured at 428 nm (pH 8) are based on the absorbance of peptide A.

Peptides	Origin of nitrated chymotrypsin	
	free enzyme	complex
A	1·00	1·00
B_1	0·45	0·34
B_2	0·55	0·43

those containing Tyr No 94 and Tyr No 228, respectively. The amino-acid sequence of the peptides are given in the legend to Fig. 2.

The relative distribution of nitrotyrosine in peptides A, B_1, and B_2 is given in Table II and shows that the peptides B_1 and B_2 together contain the same amount of nitrotyrosine as peptide A *i.e.* that tyrosine No 146 and No 171 are nitrated equally. The figures were reproducible within 10% in four independent nitration experiments.

The chymotrysin–trypsin inhibitor complex was investigated in the same way as the free enzyme. The quantities of the two components used correspond to 1·2 mol of inhibitor per mol of enzyme. Under these conditions, the residual chymotryptic activity towards acetyl tyrosine ethyl esters[13] was 25–30% of that shown by the free enzyme. Less than 10% residual activity could be detected in the presence of a 3-fold molar excess of inhibitor.

The complex was nitrated in both situations without isolation from free inhibitor, and no differences with respect to tyrosine residues were observed. A small amount of polymer which formed during nitration[14,15] was removed either by rechromatography of the nitrated complex on Sephadex G75 at pH 8, or in the dissociation step on Sephadex G75 at pH 3. When the first method was adopted, a further rechromatography yielded a single, symmetrical peak, as shown in Fig. 3. The amino-acid analysis of the nitrated complex is given in Table I; 4·0 mol of tyrosine and 2·8 mol of nitrotyrosine were found per mol of complex. Spectrophotometric measurements gave 3·8 mol of nitrotyrosine per mol of complex.

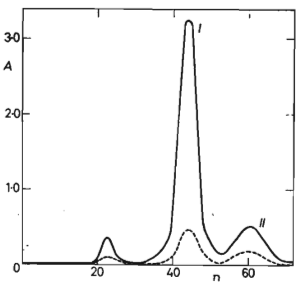

FIG. 1

Isolation of Nitrated Chymotrypsin after Dissociation of the Nitrated Complex on a Sephadex G 75 Column (1·6 × 50 cm)

I Nitrated chymotrypsin, *II* nitrated trypsin inhibitor. *A* Absorbance of fractions measured at 280 nm (full line), and at 350 nm (broken line), *n* number of fractions collected at the rate of 1·6 ml in 15 min. The nitrated complex (20 mg) was applied to the column dissolved in 1·7 ml 0·1M-NaCl, pH 3·0.

After dissociation of the nitrated complex, and separation of the two components on Sephadex G75, the elution profile shown in Fig. 1 was obtained. The amino acid composition of fraction I, given in Table I, shows that this fraction is chymotrypsin containing up to 10% inhibitor. Spectrophotometric measurements gave 2·1 mol. of nitrotyrosine per mol of chymotrypsin.

The completeness of dissociation of the complex was verified in the following way. A complex was prepared from chymotrypsin and an inhibitor containing two nitrotyrosine residues[2]. When this complex was dissociated, the absorbance of the nitrotyrosine residues (measured at 350 nm in an acid medium) was found entirely in a position corresponding to peak *II* of Fig. 1.

A peptide mixture was prepared by chymotryptic digestion of the S-sulphoderivative of chymotrypsin dissociated from the nitrated complex. The relative amounts of nitrotyrosine found in the peptides A, B_1 and B_2 after elution from peptide maps are given in Table II. These results show that the two tyrosine residues present in these peptides, Nos 146 and 171, were nitrated equally. The other two residues, Nos 94 and 228, peptides C and D respectively, were not nitrated, even after a reac-

FIG. 2

Peptide Map Prepared from the Chymotryptic Digest of Nitrated and S-sulphonated Chymotrypsin

Peptides: A Gly-Leu-Thr-Arg-Tyr^{146}, B_1 Ser-Asn-Thr-Asn-Cys-Lys-Lys-Tyr^{171}, B_2 Leu-Ser--Asn-Thr-Cys-Lys-Lys-Tyr^{171}, C Lys-Asn-Ser-Lys-Tyr^{94}, D Gly-Ser-Ser-Thr-Cys-Ser-Thr-Ser--Thr-Ser-Thr-Pro-Gly-Val-Tyr^{228}.

S Origin, first direction electrophoresis in system S_1 (left anode, right cathode), second direction descending chromatography in system S_2. Reference amino acid mixtures: 1 $CySO_3H$,Lys, Arg,Glu,Ala,Pro,Tyr,Phe,Leu, 2 $CySO_3H$,Glu,Ala,Arg.

tion time fo 200 min. Nitrotyrosine containing peptides arising from the small amount of inhibitor present were located only among the neutral peptides, and thus did not interfere with those arising from chymotrypsin.

DISCUSSION

The estimation of nitrotyrosine in protein hydrolysates has on occasions been found to present some difficulties[14-16]. Since this was also the case in the present work, emphasis was placed on the isolation from peptide maps of peptides containing nitrotyrosine, and the determination of their relative concentrations from spectrophotometric measurements. This method has the added advantage of providing direct information about individual residues.

The peptide map prepared from the chymotryptic digest of nitrated and S-sulphonated chymotrypsin differs from that reported earlier[4] in that five, and not four, tyrosine-containing peptides were found to arise from the four tyrosine residues of chymotrypsin. It was shown that peptides B_1 and B_2 both contain Tyr No 171, and that they differ as a result of chymotryptic cleavage at either one or the other of two adjacent leucine residues, No 162 and 163, in the amino acid sequence of chymotrypsin. This having been established, spectrophotometric measurements showed that Tyr Nos 146 and 171 of free chymotrypsin are nitrated equally, in agreement with the earlier work[4].

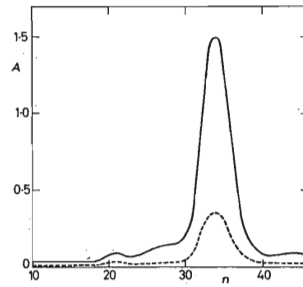

FIG. 3

Rechromatography of the Nitrated Chymotrypsin–Trypsin Inhibitor Complex on a Sephadex G 75 Colums (6·1 × 50 cm)

A Absorbance measured at 280 nm (full line) and at 428 nm (broken line), *n* number of fractions collected at the rate of 22 ml in 15 min. The nitrated complex (100 mg) was applied to the column dissolved in 29 ml 0·05M-NH_4HCO_3, pH 8·2.

When chymotrypsin was nitrated while bound in a complex with the trypsin inhibitor, the results obtained were not significantly different from those for the free enzyme. Evidently, no appreciable change in the environment of the tyrosine residues results from formation of the complex. The suggestion[1] that a tryptophan residue is involved in the interaction is thus further supported by the results of the present work. Direct evidence of this is available for the trypsin–trypsin inhibitor complex form a study of the oxidation of tryptophan residues by N-bromosuccinimide[17]. The homology between the positions of tryptophan residues in trypsin and chymotrypsin, together with examination of the three-dimensional structure of chymotrypsin[18], allow as a working hypothesis the conclusion that Trp No 215 is involved in the bond between chymotrypsin and the pancreatic inhibitor.

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