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THE ACTION OF N-BROMOSUCCINIMIDE ON TRYPSINOGEN AND ITS DERIVATIVES

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SUMMARY

The reactions of trypsin, trypsinogen, acetyltrypsinogen, and an enzymically active fragment of trypsinogen with N-bromosuccinimide have been explored. Under the conditions used, the reagent selectively oxidized the tryptophan residues without significant cleavage of tryptophyl peptide bonds. The marked difference in reactivity of tryptophan in trypsin and trypsinogen is ascribed to differences in their secondary or tertiary structure. Enzymic inactivation (trypsin) or loss of activatability (trypsinogen) was studied as a function of the oxidative modification of tryptophan. Such partially inactivated enzyme preparations still had their DFP phosphorylation sites intact. At least one tryptophan residue may be needed for activity. This demonstrates that an intact phosphorylation site *per se* is not sufficient for enzymic activity.

INTRODUCTION

The selective cleavage of C-tryptophyl peptide bonds in various model peptides, the hormone glucagon, and several proteins, *e.g.*, TMV protein and serum albumins has been reported recently. Selective cleavages varying in yield from 10–40 %, have been

observed using N-bromosuccinimide or N-bromoacetamide[1-3]. The action of NBS on trypsinogen, the precursor of the enzyme trypsin, was undertaken to see whether the zymogen upon activation after selective cleavage could lead to active fragments smaller than trypsin. In the course of this work the emphasis shifted from selective cleavage to selective oxidation of tryptophan residues and to the study of the properties of the modified trypsin and trypsinogen.

MATERIALS AND METHODS

Trypsinogen (1 × crystallized) and trypsin (2 × crystallized, either salt free or lyophilized) were purchased from the Worthington Biochemical Corporation, Freehold, N. J. Crystalline insulin was a gift from Dr. O. K. BEHRENS of the Eli Lilly Company, Indianapolis, Ind. N-bromosuccinimide was obtained from the Arapahoe Chemical Co., Boulder, Colorado, and recrystallized from water before use. *p*-nitrophenyl acetate was obtained from Dr. G. L. SCHMIR and recrystallized from aqueous ethanol, and ether–petroleum ether, m.p. 78–79° (reported[4] m.p. 79.5–80.0°). Benzoyl-L-arginine ethyl ester was purchased from the Mann Research Laboratories, New York.

The oxidations of trypsin and trypsinogen were carried out by slowly adding an aqueous solution of NBS (approx. 1 μmole/ml) with constant swirling, to 0.1 % solutions of the proteins in 0.1 *M* acetate buffer pH 4, until the desired amount of NBS had been added. The instantaneous oxidation of tryptophan was followed in a Cary self-recording spectrophotometer by the decrease of absorbance at 278 mμ. The extent of the oxidation of tryptophan was calculated on a molar basis by multiplying the decrease in extinction by the empirical[2] factor 1.31, using 5500 as the molar extinction coefficient for tryptophan.

The activation of NBS-treated trypsinogen samples was performed as follows: The protein solution at pH 4 was brought to pH 8.0 by addition of the required amount of alkali, and a concentrated borate buffer solution containing $CaCl_2$ was added to obtain a final molarity of 0.1 *M* and 0.02 *M* in borate and calcium ions, respectively. The activation was initiated by the addition of a catalytic amount of trypsin (1 part of enzyme to 100 parts of zymogen).

Activities were measured using benzoyl-L-arginine-ethyl ester (BAEE) as substrate according to the spectrophotometric procedure of SCHWERT AND TAKENAKA[5]. The activation of trypsinogen samples, with or without NBS treatment, was followed also by the measurement of alkali consumed during the process with the aid of an automatic titrator (Type TTT 1a, Radiometer, Copenhagen) coupled with a recorder (Ole Dich, Copenhagen).

Oxidized insulin was prepared by the performic acid oxidation procedure of SANGER[6]. The activity of enzyme samples on oxidized insulin as substrate was followed by determination of the liberated α-amino groups by reaction with ninhydrin[7].

The reaction of *p*-nitrophenylacetate (NPA) with NBS-treated trypsin samples was followed by the appearance of the *p*-nitrophenoxide absorption at 400 mμ. The reaction was started by the addition of 0.1 ml NPA solution (in ethanol) to 3.0 ml

Abbreviations used are: NBS, N-bromosuccinimide; BAEE, benzoyl-L-arginine ethyl ester; NPA, *p*-nitrophenylacetate; DFP, diisopropylphosphofluoridate; DIP, diisopropylphosphoryl; DNP, dinitrophenyl.

of enzyme sample in 0.1 *M* phosphate buffer pH 7.6. The final concentration of NPA in the reaction mixture was $5 \cdot 10^{-4}$ *M*. Appropriate controls to correct for the spontaneous hydrolysis of NPA were included in the experiments.

The diisopropylphosphoryl (DIP) derivatives of various enzyme samples were prepared by treatment with a 50-fold excess of diisopropylphosphofluoridate (DFP) at pH 8.0 in the presence of 0.02 *M* calcium chloride. After 6 h no enzymic activity was left. The protein was precipitated by the addition of solid ammonium sulfate to saturation. The precipitate was collected by centrifugation, dissolved in the minimum of water and dialysed against 0.001 *N* hydrochloric acid for 3–4 days, with repeated changes of the dilute acid. The dialysed solutions were lyophilized.

The phosphorus content of DIP-proteins was determined by the A.O.A.C. Micro Phosphorus Method[8], as modified in the Analytical Service Laboratory of this Institute by the use of perchloric acid for rapid wet ashing. Protein concentrations were calculated from absorbance at 278 mμ of the solutions using a conversion factor of 0.6 for unoxidized samples, and suitable calculated factors in the case of NBS-treated samples. The protein concentrations so determined were confirmed by actual nitrogen determinations according to the procedure of LANNI *et al.*[9].

N-Terminal groups were analyzed by the 2,4-dinitrofluorobenzene method according to established procedures[10].

RESULTS

Oxidation of tryptophan

The addition of NBS to trypsinogen in acetate buffer at pH 4 led to a proportional and instantaneous decrease of tryptophan absorption at 278 mμ. The tryptophan content was calculated on the basis of the maximum decrease in absorbance at 278 mμ and gave 3.96 tryptophans[11] per molecule of trypsin (mol. wt. 24,000)[12]. With trypsinogen the indole absorption disappeared after the addition of 1.8 moles of NBS per mole of tryptophan. This is in keeping with the average consumption of NBS (1.5–1.6 moles) for model tryptophyl peptides. The use of the same technique on trypsin gave 3.84 tryptophans per molecule. However, in this case the reaction with NBS was not as specific as with trypsinogen, since 3.2 moles of NBS/mole of tryptophan were required to eliminate tryptophan absorption at 278 mμ. It has been shown in experiments with model compounds that tyrosine is not oxidized before tryptophan at pH 4*. Furthermore, the amino acid compositions of acid hydrolysates of trypsin and oxidized trypsin (3 % activity) were the same within experimental error (Table I). Curves showing the tryptophan destruction in the two proteins are given in Fig. 1. The products to be expected from oxidation of tryptophan in this manner are derivatives of oxindolylalanine.

Influence of NBS on the activation of trypsinogen

Trypsinogen exposed to pH 4 but not subjected to the action of NBS showed the typical activation phenomenon[13] on treatment with trypsin at pH 8. Treatment of the zymogen with NBS however, altered the activation characteristics, the nature of the change being dependent on the amount of NBS used. Fig. 2 shows that in-

* Unpublished experiments by Dr. A. PATCHORNIK and Dr. G. L. SCHMIR.

TABLE I

AMINO ACID COMPOSITION OF TRYPSIN AND OXIDIZED TRYPSIN

Salt-free trypsin (Worthington Biochemical Corporation Lot No. 688SF) unoxidized, and oxidized as described in the text, were hydrolyzed by constant-boiling hydrochloric acid for 20 h at 105°. Except for the oxidation step, the samples were treated identically. The oxidized sample possessed 3 % of the original activity. The hydrolysates were analyzed for amino acids using the Automatic Amino Acid Analyzer (Beckman-Spinco) developed by SPACKMAN, STEIN AND MOORE[13]. 99 and 97 % of the applied nitrogen was recovered in the case of unoxidized and oxidized trypsin hydrolyzates respectively.

Amino acids	*Trypsin*		*Oxidized trypsin*	
	Number of residues per 24,000 g protein			
	Observed	*Probable*	*Observed*	*Probable*
Aspartic acid	22.0	22.0	22.2	22.0
Threonine	9.6	10.0	9.7	10.0
Serine	32.4	32.0	31.7	32.0
Glutamic acid	14.2	14.0	13.9	14.0
Proline	8.20	8.0	7.9	8.0
Glycine	25.0	25.0	25.1	25.0
Alanine	14.2	14.0	14.2	14.0
Half cystine	11.5	12.0	11.4§	12.0
Valine	11.8	12.0	11.9	12.0
Methionine	1.20	1.0	1.25	1.0
Isoleucine	12.3	12.0	12.4	12.0
Leucine	14.1	14.0	14.0	14.0
Tyrosine	9.0	9.0	8.5	8–9
Phenylalanine	3.02	3.0	2.93	3.0
Lysine	13.7	14.0	13.8	14.0
Histidine	3.10	3.0	3.10	3.0
Ammonia	29.3	29.0	29.0	29.0
Arginine	2.05	2.0	2.1	2.0

§ A very small amount of cysteic acid (0.07 mole/24,000 g protein) was observed in the chromatogram of oxidized trypsin hydrolysate.

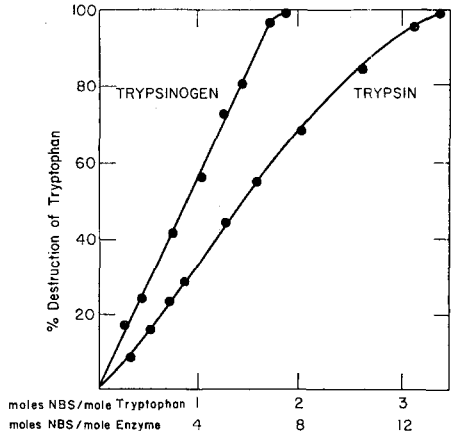

Fig. 1. Comparison of the effects of N-bromosuccinimide on the disappearance of tryptophan absorption in trypsinogen and trypsin.

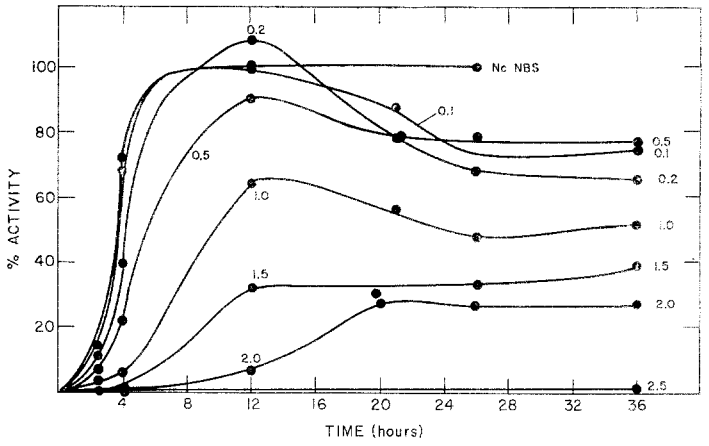

Fig. 2. Influence of various amounts of N-bromosuccinimide (moles/mole of zymogen) on the time and extent of autoactivation of trypsinogen at 25°, borate buffer pH 8 and 0.02 M Ca^{++}.

creasing quantities of NBS progressively lowered the extent of maximal activity. With 1.0, 1.5, 2.0 and 2.5 moles of NBS/mole of zymogen, maximum activities corresponded to 60, 45, 30 and 3 % of the control. In addition to the decrease in activity, NBS treatment also resulted in a retardation of activation, dependent on the amount of NBS used. This lag in activation was also observed on very limited oxidation (0.1 to 0.5 moles of NBS), although the same final activity as in the control was obtained. Likewise, studies on the alkali consumption during the activation of such trypsinogen samples revealed this delay. The catalytic amount of trypsin added to initiate the activation process cannot be affected by NBS, since all of the reagent is quantitatively consumed at the time of addition. Succinimide, the end product formed from NBS, was found to have no effect, either on the activation process or on the activity of trypsin. The possibility of the formation of a competitive trypsin inhibitor during the oxidation is eliminated, since oxidized trypsinogen does not interfere with the activation of intact trypsinogen. The appearance of activity in the oxidized trypsinogen samples parallels the proteolysis during activation, followed by the uptake of alkali. The lag apparently is related to the protein becoming less susceptible to the proteolysis essential to activation. Aggregation of the partially oxidized zymogen is a possible explanation for the retardation of the proteolysis.

The relationship between the oxidation of tryptophan and the maximal activity is shown in Fig. 3. The two curves were obtained in experiments in which the protein concentrations differed. In general, it was found that the more concentrated the protein solution, the greater the effect of a given amount of NBS. However, Fig. 3 shows that nearly 18 % of the tryptophan can be oxidized without much effect on the activatability, which falls off rapidly with the oxidation of a second tryptophan.

The influence of NBS on acetyltrypsinogen, which can be converted into an active fragment (mol. wt. approx. 6000) by the action of pepsin[15, 16], was also investigated. It resulted in a stepwise destruction of the activity obtained on peptic activation. Treatment of the acetylzymogen with 1.0, 2.0 and 3.0 moles of NBS led to values of 25, 13 and 3 % compared with the activities in control experiments. In this case, as well as with the purified active fragment, the oxidation of tryptophan

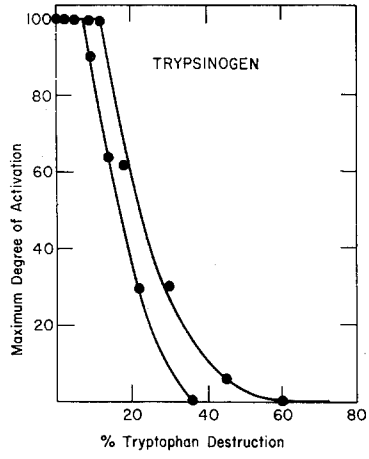

Fig. 3. Two representative runs (different volumes and concentrations) on the effects of progressive oxidation of tryptophan on the final enzymic activity of trypsinogen.

could not be followed spectrophotometrically because of precipitation of protein. The addition of 1.5 moles of NBS to the active fragment caused total and irreversible inactivation. End terminal analysis led to a single new DNP-amino acid, di-DNP-lysine in 3 % yield, probably by cleavage of the one tryptophan peptide bond present*.

Effect of NBS on trypsin

When a 0.1 % solution of trypsin in acetate buffer at pH 4 was treated with increasing quantities of NBS, no significant effect on the enzymic activity was noticed up to 3.0 moles of NBS/mole of protein. Further oxidation inactivated the enzyme. With 4.5 and 7.0 moles of NBS, 65 and 92 % of the original activity was lost. Even after treatment with 10 moles of NBS, about 2 % of residual activity was still de-

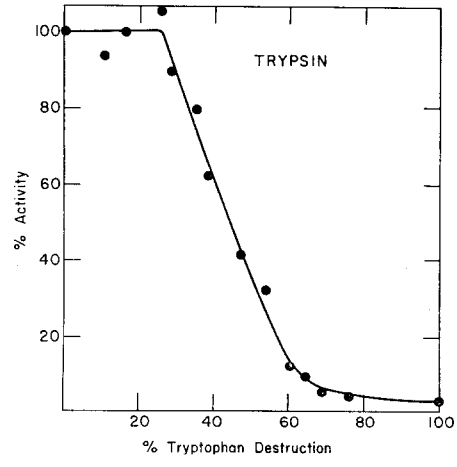

Fig. 4. The decrease of enzymic activity of trypsin as a function of the disappearance of the tryptophan chromophore caused by NBS.

* A low yield in the cleavage reaction does not necessarily mean that the new end group is spurious. Often the yields increase when the right conditions are achieved (*cf.* ref. 3).

tectable. The relationship between the oxidation of tryptophan and the loss in tryptic activity is shown in Fig. 4. This curve is similar to that for trypsinogen (Fig. 3).

In studies on the hydrolysis of *p*-nitrophenyl acetate (NPA) by active and partially NBS-inactivated samples of trypsin, the activities towards NPA roughly paralleled activities towards BAEE. However, the "initial burst"[17] of *p*-nitrophenol was not observed, and this was the case with all samples, even the untreated control, which was merely exposed to an 0.1 *M* pH 4 acetate or phosphate buffer.

With oxidized insulin as substrate, a progressive drop in activity of oxidized trypsin samples was observed. In all cases, the extent of cleavage was restricted to the two known sites for tryptic cleavage in the insulin molecule.

The optimal pH for BAEE hydrolysis was not changed on partial oxidative inactivation of trypsin.

The incorporation of phosphorus

Enzyme preparations from oxidized trypsinogen of varying degrees of hydrolytic activity were allowed to react with DFP and the amounts of incorporated phosphorus determined. Irrespective of the activity values reached after complete activation, one mole of phosphorus per mole (24,000 g) of protein was incorporated. A partially activated sample, having approx. 3 % activity as compared with 25 % on maximum activation, contained only 0.17 mole of phosphorus. After complete activation this value increased to 1 mole (Table II).

Oxidized trypsin samples, inactivated to various degrees, were also reacted with DFP. Samples of low tryptic activity still bound one mole of phosphorus per mole

TABLE II

THE INCORPORATION OF PHOSPHORUS ON DFP TREATMENT OF SAMPLES OF TRYPSINOGEN AND TRYPSIN AFTER OXIDATION WITH NBS

No.	*Enzyme*	*Moles of NBS per mole of protein**	*Percent tryptophan destruction*	*Percent activity observed***	*Moles of phosphorus per mole of protein*
1	Trypsinogen	0	0	100	1.04 (1.06)***
2	Trypsinogen	1.5	17	50	0.98 (0.98)
3	Trypsinogen§	2.0	30	3–5; 25§§	0.17 (0.18) (1.07)§§
4	Trypsin	0	0	100	0.90 (0.92)
5	Trypsin	3.6	36	50	1.0 (0.91)
6	Trypsin	8.0	77	3	0.61 §§§
7	Trypsin	6.0	62	approx. 10	0.37† (0.37)

* The molecular weight of trypsinogen and trypsin is assumed to be 24,000.

** In the case of trypsinogen activation was obtained in the presence of catalytic amounts of trypsin.

*** The values in parentheses represent ratios in which the protein values were obtained by spectrophotometric methods by the measurement of absorbance at 280 mμ and the use of empirical conversion factors, while for the other values the protein figures were determined by nitrogen analyses.

§ When a large volume of a 0.2 % solution of trypsinogen was treated with 1.5 and 2.0 moles of NBS, 24 and 48 h, respectively, were required to obtain the maximal activity (50 % and 25 %). The DFP inhibition was studied with NBS-oxidized trypsinogen at two different intervals of activation.

§§ DFP treatment of fractions with only 3–5 % sub-maximal activity resulted in an incorporation of 0.17 mole of phosphorus, which increased to 1.07 moles at maximum activity (25 %).

§§§ The time of reaction with DFP was 20 h.

† The time of reaction with DFP was 6 h.

of protein. A sample which had 10 % activity incorporated 0.37 mole of phosphorus after 6 h of reaction with DFP, but a sample with only 3 % activity incorporated 0.61 mole after 18 h (Table II).

The reaction with DFP was carried out under conditions so as to minimize the phosphorylation of the second reactive site in the protein[18]. The oxidative inactivation is not reversible at pH 8, under the condition used for phosphorylation, since suitable controls which were kept at the same pH failed to show any increase in activity towards BAEE on prolonged standing.

End group analysis

Qualitative analysis for N-terminal groups on an NBS inactivated sample of trypsin showed only one major DNP-amino acid spot, corresponding to DNP-isoleucine, from the known N-terminus of trypsin[19]. However, the failure to find any other spots in this experiment does not rule out the formation of minor amounts of new N-terminal groups due to tryptophyl peptide cleavage[1-3]; it only indicates that under the inactivation conditions extensive cleavage does not occur. N-Terminal analysis after treatment of trypsinogen with twelve moles of N-bromoacetamide in 70 % acetic acid yielded DNP-amino acid spots corresponding to DNP-phenylalanine (about 20 %) and di-DNP-lysine (about 3 %), in addition to the DNP derivative of valine, the known N-terminus of trypsinogen[19].

DISCUSSION

Treatment of trypsinogen and trypsin with NBS results in the largely selective oxidation of the tryptophan residues in these proteins, as is revealed by the spectral changes, and supported by amino acid analyses (Table I). The relative resistance of the tryptophyl residues in trypsin is probably a manifestation of overall structural differences between the zymogen and the enzyme. Nearly 18 and 25 % of the four tryptophan residues in the zymogen and the enzyme, respectively, can be destroyed without impairing the catalytic activity (potential or present). This suggests that one tryptophan residue is more accessible to oxidation than the other three, and is not essential for enzymic activity. The manner in which enzymic activity is lost during further oxidation (*cf.* Figs. 2 and 3) suggests the implication of at least one of the remaining three tryptophan residues in the maintenance of catalytic activity. The active fragment (mol. wt. approx. 6000) from acetyltrypsinogen contains but one tryptophan of the four originally present. Since oxidation of this particular tryptophan inactivates the enzyme, the part of the protein molecule containing this residue probably is essential for enzymic activity.

The residual activity is an inherent property of the oxidized proteins and is not due to intact starting material. The following observations support this view:

(a) The specific activity of various NBS treated trypsins could not be altered by fractional precipitation with ammonium sulfate. (b) Enzyme samples which had lost considerable activity towards BAEE were capable of incorporating one mole of phosphorus (from DFP) per mole of protein; this rules out unoxidized protein as a source for residual activity. Trypsin which had lost nearly 98 % of its activity towards BAEE could still incorporate 0.61 mole of phosphorus, a value which is nearly 30 times the expected uptake if the residual 2 % activity were due to intact trypsin.

(c) The DIP-derivative of oxidized trypsin (which had 50 % activity prior to treatment with DFP) was found to be homogeneous in the ultracentrifuge, and to sediment at the same rate as the DIP-derivative of unoxidized trypsin.

The oxidation of trypsin by NBS affects its activity towards BAEE, a typical substrate, without impairing its ability to react with DFP. The phosphorylation site which is currently considered to be a part of the "active site" of the enzyme molecule[20-22] apparently remains unaffected during the oxidation. This observation demonstrates the necessity of other factors besides an intact phosphorylation site for the optimal function of the enzyme. Similarly, WOOD AND BALLS[23] have shown that enzymic oxidation of a single tryptophan in chymotrypsin lowered its catalytic activity to 50 % without affecting its ability to be phosphorylated. They considered tryptophan to be an auxiliary group, necessary for full activity. The inactivation may be the result of a slight disorientation* of the active configuration of the protein molecule, brought about by the modification of one or more tryptophan residues. The actual role of tryptophan in relation to enzymic activity remains to be elucidated.

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