Studies on the Chemical Modification of Trypsin

S. I. Ahmad

Department of Pharmacy, University of Karachi, Karachi

(Z. Naturforsch. **29 c**, 136–138 [1974]; received October 15, 1973)

Dibenamine, Trypsin

Dibenamine (*N*,*N*-dibenzyl-β-chloroethylamine) a potent adrenergic blocking agent inhibits trypsin in an irreversible manner. Electrophoresis and peptide mapping (finger printing) have shown significant changes in the number of peptides and their mobility.

Introduction

Many effects of dibenamine (*N*,*N*-dibenzyl-β-chloroethylamine) on physiological systems have been reviewed by Graham [1] and Nickerson [2]. However their effect on enzyme activity was not reported till Beddoe and Smith [3] observed that dibenamine also reacts with acetylcholinesterase under rather specific conditions in an irreversible manner to give an inactive modified enzyme. The inhibiting compound for the reaction is probably the ethyleniminium ion which is present in high concentration in solutions of dibenamine hydrochloride which are normally acid (pH 3.2). Reaction between alkylating compounds and the enzyme does not occur in the acid solution but occurs in near neutral media where a functional group on the enzyme is in the correct state of ionization. The alkylating compound is very unstable at near neutral pH and decomposes within a minute or so. Consequently inhibition is only observed when either the dibenamine solution is added to the enzyme at near neutral pH or an acidic mixture of the two is rapidly raised to near neutral pH. The present study was undertaken to determine the effects of inhibition on the structure of trypsin.

Materials and Methods

The assay of native and inhibited trypsin was carried out essentially by the method of Schwert and Tanaka [4] using *N*-benzoyl-L-arginine ethyl ester (BAEE) as substrate.

Inhibition of trypsin by dibenamine: A solution of dibenamine hydrochloride (2 ml; $7.5 \cdot 10^{-4}$ M) containing sodium chloride (0.2 M) was added to the assay mixture at pH 7.8 containing trypsin (10 μg). The pH of the mixture was readjusted and it was incubated for 15 min. A solution of BAEE (2 ml, $7.5 \cdot 10^{-4}$ M) was then added and assay continued in the usual manner. The enzyme showed no activity over a period of 20 min.

Inhibition of trypsin by dibenamine in bulk: A solution of trypsin (20 ml; 200 μg) was added to a solution of dibenamine hydrochloride (20 ml; $1.5 \cdot 10^{-3}$ M) and pH of the mixture raised to 7.8 by the rapid addition of dilute alkali. Aliquots (2 ml; 4 ml and 8 ml) were separately added to the assay mixture in the assay vessel and the enzyme activity was measured in the usual manner. Control experiments were conducted in the absence of dibenamine hydrochloride.

Dialysis of native and inhibited trypsin: Solutions of native and inhibited trypsin were dialysed over a period of 60 hours. Separate samples were taken at suitable intervals of time and assayed for enzyme activity in the usual manner.

Preparation of peptide maps (finger prints) of native and inhibited trypsin: A sample of lyophilized inhibited enzyme (6 – 8 mg) from which excess dibenamine had been removed by diafiltration was dissolved in a solution of ammonium bicarbonate (0.5 ml; 0.1 M; pH 8.7). This solution was mixed with trypsin (10 μg) in hydrochloric acid (1 ml; 10^{-3} M) and stored at room temperature for 3 hours. At the end of the period a drop of glacial acetic acid was added and the solution was lyophilized to remove ammonia. The sample was then dissolved in pyridine-acetic acid buffer (1.25%) pH 4.7 and applied on a sheet of 116 cm long 3 MM Whatman filter paper. The sample was applied in a streak at one corner of the filter paper and then the solution of the same buffer was spread using a wash bottle over the whole of the paper with careful avoidance of the sample streak. The excess of the buffer was then removed from the wet paper by blotting. The sheet supported by a glass rod was transferred with a side ways movement to prevent the paper floating. The high voltage electro-

Requests for reprints should be sent to Dr. S. I. Ahmad, Department of Pharmacy, University of Karachi, University Campus, *Karachi-32*, Pakistan.

phoresis apparatus was supplied by Gilson Medical electronics, France, 69 Rue Gambatta, 95 Villier le Bel. The sheet was set in such a manner that the portion with the sample spot stayed on the left side of the equipment. The lid of the chamber was then closed and electrophoresis was conducted for $3\frac{1}{2}$ hours at 3500 V. At the end of the run the paper was removed and dried completely in an oven at 60 °C for about 2 – 3 hours. The chromatogram was run using Hills modified buffer (200 ml *n*-butanol 38%; acetic acid 8%; pyridine 27% and water 27%). After 12 hours the sheets were removed from the tank and dried at 60 °C. The sheets were then dipped in a solution of ninhydrin (0.2%) in acetic acid to develop the spots and then reduced at 60 °C. The chromatograms were photographed and finally stored in the dark. The photographs of the chromatograms are reproduced in Figs 1* and 2.

Results and Discussion

Addition of dibenamine to trypsin at pH 7.8 results in the complete inhibition of trypsin, as shown in Table I. The reaction between trypsin and dibenamine had similar characteristics to that previously noted by Al-Shabibi [5] for chymotrypsin and dibenamine. The reaction reached completion very rapidly and appeared to be a titration of the enzyme by an alkylating agent since the same amount of enzyme was inhibited by a fixed concentration of dibenamine independent of enzyme concentration.

Table I. Effect of incubation of dibenamine on the extent of reaction with trypsin.

Period of incubation of dibenamine at pH 7.8 [min]	[% Enzyme activity remaining]
Immediate addition of enzyme	00.0
5	96.0
20	96.0

Dialysis of inhibited enzyme in near neutral media for a period of 60 hours did not lead to the regeneration of the enzyme activity during this period. Control experiments with the native enzyme showed that the enzyme was stable under these conditions for 40 hours but there was a loss of activity after 60 hours (Table II). Therefore it was concluded that dibenamine inhibits trypsin in an irreversible manner and a modified trypsin is produced. To investigate the effect of dibenamine on the structure of trypsin, peptide maps of trypsin and inhibited trypsin were prepared. Although peptide maps for a number of enzymes are known [6, 7] a peptide map for native trypsin has not been reported previously. Trypsin contains fourteen lysine and three arginine residues and it was expected that on trypsin digestion 18 spots each corresponding to a peptide fragment would be produced. In our studies the trypsin peptide map was very clear (Fig. 1). It showed the separation of 18 main fragments with some degree of overlapping due to high loading. This map was designated as the "reference map" in the following discussion.

Table II. Effect of dialysis on the native and inhibited enzyme.

Period of dialysis [hours]	Native enzyme: [% of enzyme activity remaining]	Inhibited enzyme: [% of enzyme activity remaining]
20	100.0	Nil.
40	96.0	Nil.
60	87.0	Nil.

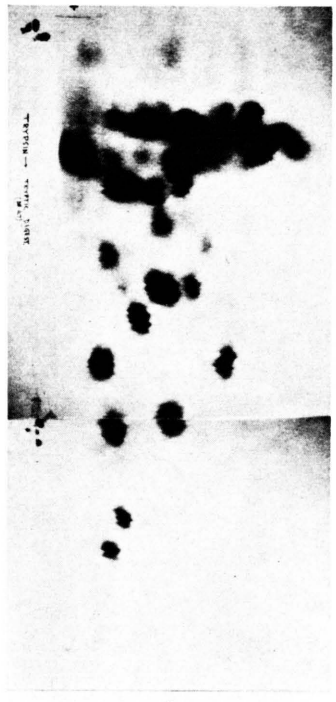
The peptide map obtained from the dibenamine inhibited trypsin (Fig. 2) on comparison with the reference map showed one or two additional fragments than were previously obtained. This further fragmentation of the enzyme molecule can be explained by postulating that a few more susceptible fragmentation points occur on the dibenamine-inhibited enzyme molecule. This situation might have arisen either through a change in conformation of the protein molecule prior to enzyme hydrolysis or by a change in the net charge present on the molecule due to alkylation by dibenamine of some of the available amino acid residues, especially the charged polar residues.

Comparison of the electrophoretic displacement of the peptide on the maps for the inhibited and native enzyme showed greater displacement of some of the peptides on the former which means that the mobility of a few peptides had changed. This is expected if the alkylated peptides posses a more positive charge on balance than the corresponding peptides from the native enzyme. The change in the mobility of a number of peptides shown by the finger prints of the inhibited enzyme suggests that dibenamine has alkylated the number of amino acid residues present in enzyme molecule.

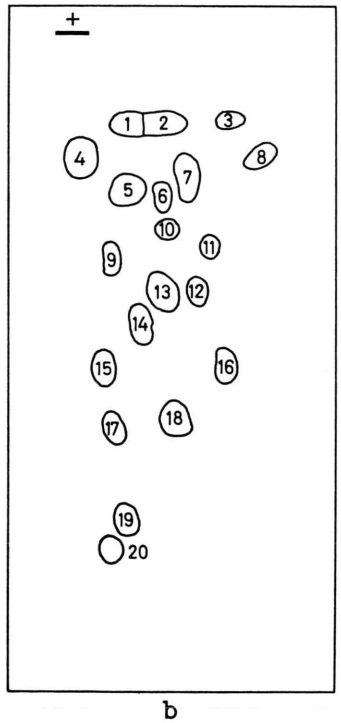
* Figs 1 and 2 see Table on page 138 a.

The author wishes to express his gratitude to Prof. Dr. G. v. Ehrenstein, Director, Department of Molecular Biology, Max-Planck Institute for experimental Medicine, D-3400 Goettingen, West Germany, for providing facilities of his laboratories to carry out this work.

[1] J. D. P. Graham, Progress in Medicinal Chemistry, **Vol. II**, (eds. G. P. Ellis and G. B. West), p. 132, Butterworth, London 1962.
[2] M. Nickerson, Pharmacological Basis in Therapeutics, (eds. Goodman and Gillman), p. 550.
[3] F. Beddoe and H. J. Smith, J. Pharmacy and Pharmacol. **23**, 77 [1971].
[4] G. W. Schwert and Y. Tanaka, Biochim. biophysica Acta [Amsterdam] **15**, 570 [1955].
[5] H. Al-Shabibi, Ph. D. Thesis, University of Wales, U. K. 1972.
[6] C. B. Anfinsen, S. E. G. Aquist, J. P. Cooke, and B. Jonsson, J. biol. Chemistry **234**, 1118 [1959].
[7] L. B. Smillie and B. S. Hartley, J. molecular Biol. **10**, 183 [1964].



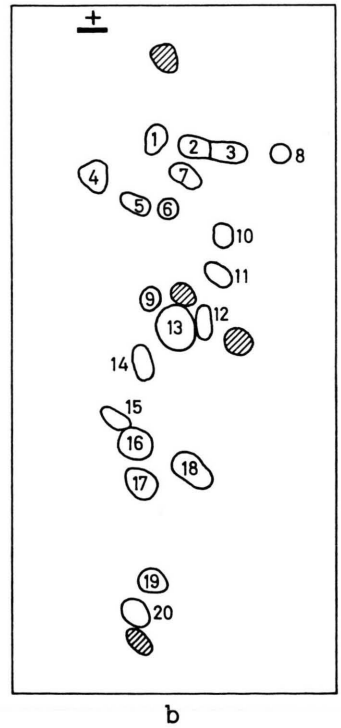
a



b

Fig. 1. Finger print of native enzyme, a. original photograph, b. diagrammatic representation.

a



b

Fig. 2. Finger print of trypsin inhibited by dibenamine, a. original photograph, b. diagrammatic representation (extra peptides are shaded).