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Inhibition of α-chymotrypsin by dibenamine

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α-Chymotrypsin is rapidly inhibited by an acid solution of dibenamine in an irreversible manner when the reactants are mixed in a buffer at near neutral pH. The degree of inhibition depends on the molar ratio of the reactants. The alkylating species is probably the ethyleniminium ion from dibenamine, this ion being present at a higher level in the acid media than in the near neutral media used here.

Dibenamine reacts in an irreversible manner with a number of drug receptors, e.g. muscarinic (Furchgott, 1954), α-adrenergic (Nickerson & Goodman, 1946) and histaminic (Graham, 1962), and although a potential candidate for the labelling and isolation of receptor material from these receptors such studies have been unrewarding (Takagi, Akao & Takahashi, 1965; Takagi & Takahashi, 1968). We have applied mass spectrometry to the identification of the alkylated residues present in the enzyme ribonuclease after reaction with iodoacetate (Ahmad & Smith, 1973) and consider that this technique could be similarly applied to a pharmacological drug receptor system which has been alkylated with a suitable agent. We have studied the inhibition between dibenamine and a model protein of known structure, α-chymotrypsin, and the characteristics of this reaction are reported here.

EXPERIMENTAL AND RESULTS

Materials. The α-chymotrypsin used was from bovine pancreas and had been recrystallized three times (Koch-Light). A stock solution ($1{\cdot}95 \times 10^{-4}$M) was prepared in deionized water and stored at 4°. *N*-Acetyl-L-tyrosine ethyl ester (ATEE) solutions (0·01M) were prepared in deionized water by warming and stored at 4°.

Enzyme assay. α-Chymotrypsin was assayed in the pH-Stat (Radiometer, Copenhagen) essentially by the method of Schwert, Neurath & others (1948). The enzyme solution (2 ml) was added to a mixture of potassium dihydrogen orthophosphate buffer, pH 7·4 (2 ml, 0·02M), sodium chloride (2 ml, 1M) and deionized water (13 ml) in a jacketed assay vessel maintained at 25° under an atmosphere of carbon dioxide-free nitrogen. The ATEE (2 ml, 0·01M) was then added and the acid produced automatically titrated with sodium hydroxide solution (N/75) by the pH-Stat to maintain pH 7·4 in the assay vessel. The enzyme activity was obtained from the slope of the trace of the recorder over the initial 4 min period of the reaction.

Inhibition of α-chymotrypsin by dibenamine. A solution of dibenamine hydrochloride ($7{\cdot}5 \times 10^{-4}$M) which is normally acid (pH 3·85) containing sodium chloride (0·2M) was incubated at 25° for 10 min which is a sufficient period for maximum conversion to the inhibiting species. A solution of α-chymotrypsin (2 ml, 1×10^{-7}M) was added to the phosphate buffer mixture in the assay vessel and was immediately followed by the dibenamine solution (2 ml). ATEE was then added and the assay was carried out in the usual manner.

In similar experiments the solutions of dibenamine hydrochloride were previously adjusted with dilute alkali or dilute hydrochloric acid to pH 3·0, 5·0, 6·0, and 7·5 incubated for 20 min and then used.

Addition of dibenamine hydrochloride solution (pH 3·0, 3·85) to the enzyme in the assay vessel at pH 7·4 gave a high degree of inhibition (50%). Adjustment of the dibenamine hydrochloride solutions to pH 5·0, 6·0 and 7·5 before addition to the enzyme gave 21, 13 and 9% inhibition respectively.

Stability of the inhibiting species at pH 7·4. Dibenamine hydrochloride solution ($7{\cdot}5 \times 10^{-4}$M) containing sodium chloride (0·2M) was adjusted to pH 3 with dilute hydrochloric acid and an aliquot (2 ml) added to the phosphate buffer mixture in the assay vessel. α-Chymotrypsin (2 ml, $1{\cdot}0 \times 10^{-7}$M) was then added immediately to the vessel and after an incubation of 5 min the assay procedure was continued. In other experiments the interval between addition of dibenamine and enzyme solutions to the assay vessel was increased.

The addition of the enzyme solution to the assay vessel immediately after addition of the dibenamine solution gave complete inhibition. Delay led to very little inhibition.

Dialysis of the inhibited enzyme mixture. A solution of the inhibited enzyme was acidified with dilute acid to pH 4·4 and the solution dialysed against several changes of water (pH 4·4) for 72 h at 4°. Aliquots were then withdrawn from the sacs and assayed for enzyme activity. A control experiment was conducted in the absence of dibenamine.

After dialysis of the inhibited enzyme for 72 h, the preparation remained inactive showing that the inhibition was irreversible. A control experiment with native enzyme showed little loss of activity under the acid conditions used for the dialysis.

In another experiment the previous experiment was repeated except that the pH of the reaction mixture was not allowed to exceed pH 4 at any stage during the experiment. The dialysed protein had 53% of the original enzyme activity which confirmed that the inhibition reaction occurred to only a limited extent in acid media.

Influence of enzyme concentration on the reaction with dibenamine. Dibenamine hydrochloride solutions (19 ml, $7{\cdot}5 \times 10^{-4}$M) containing sodium chloride (0·2M) were adjusted to pH 3 and separately mixed with α-chymotrypsin (1 ml, $2{\cdot}0 \times 10^{-6}$ to $1{\cdot}6 \times 10^{-5}$M). Aliquots of the reaction mixture were assayed. An approximately constant amount of enzyme was inhibited by the dibenamine solutions when the mixture was raised to pH 7·4 on being added to the assay vessel (Table 1).

Influence of dibenamine concentration on the reaction with α-chymotrypsin. Solutions containing dibenamine hydrochloride (19 ml, $0{\cdot}75 \times 10^{-4}$ to $7{\cdot}5 \times 10^{-4}$M) and sodium chloride (0·2M) were separately adjusted to pH 3 and mixed with an α-chymotrypsin solution (1ml, $2{\cdot}0 \times 10^{-6}$M). Aliquots of each reaction mixture were removed and assayed periodically. The degree of inhibition of the enzyme was dependent on the concentration of the dibenamine solution (Table 1) although this was not a linear relation.

Influence of sodium thiosulphate on the inhibition reaction. A mixture of dibenamine hydrochloride (18 ml, $1{\cdot}9 \times 10^{-4}$M) and sodium thiosulphate (1 ml, 5×10^{-2}M) was adjusted to pH 4·2 and then incubated at 25° for 10 min. A solution of α-chymotrypsin (1 ml, $0{\cdot}5 \times 10^{-6}$M) was then added and the mixture assayed in the usual manner. Control experiments were carried out, (1) in the absence of dibenamine,

Table 1. *Influence of the relative concentrations of α-chymotrypsin and dibenamine on the inhibition reaction.*

Dibenamine concn (M)	α-Chymotrypsin concn (M)*	Enzyme activity remaining (%)
$0{\cdot}75\times10^{-4}$	$1{\cdot}0\times10^{-7}$	96
$3{\cdot}7\times10^{-4}$	$1{\cdot}0\times10^{-7}$	49
$7{\cdot}5\times10^{-4}$	$1{\cdot}0\times10^{-7}$	0
$7{\cdot}5\times10^{-4}$	$2{\cdot}0\times10^{-7}$	0
$7{\cdot}5\times10^{-4}$	$4{\cdot}0\times10^{-7}$	57
$7{\cdot}5\times10^{-4}$	$8{\cdot}0\times10^{-7}$	78

* Concentration in reaction vessel.

(2) where the thiosulphate was replaced by sodium chloride (60×10^{-2}M). The presence of the thiosulphate ion in the inhibition reaction mixture decreased the extent of the inhibition reaction and the enzyme showed about 33% of its original activity. The effect of the thiosulphate on the inhibition reaction was not due to an ionic effect since sodium chloride at the same ionic strength (60×10^{-2}M) had no effect on the inhibition reaction.

DISCUSSION

A fast inhibition reaction occurred when either a solution of dibenamine hydrochloride, which is normally acid, was added to a solution of α-chymotrypsin in near neutral media or the two solutions were mixed and the pH of the mixture was rapidly raised to near neutral pH by the addition of alkali. The enzyme was inhibited in an irreversible manner as shown by dialysis studies. The inhibition reaction possessed certain unusual features which were not apparent in the previously observed irreversible inhibition reactions between acetylcholinesterase (Beddoe & Smith, 1971), Na^+—ATPase (Roufogalis & Belleau, 1969) and dibenamine.

Using molar ratios of enzyme and inhibitor which gave complete inhibition of the enzyme when the dibenamine hydrochloride solution was added to the enzyme in buffer at near neutral pH, it was found that reversal of the addition of the reactants led to a loss in the ability of the inhibitor to inhibit the enzyme when addition of enzyme was delayed. Storage of the dibenamine hydrochloride solution at pH 5, 6 or 7·5 before addition to the enzyme similarly led to a loss in its inhibitory capacity.

Although the molar ratio of α-chymotrypsin and dibenamine hydrochloride normally employed in the reaction mixture was about 1:7000, so that a large excess of the inhibitor was present, the extent of the inhibition was influenced by deviation from this molar ratio. It was found that the degree of inhibition of the enzyme by a fixed concentration of inhibitor decreased as the enzyme concentration was increased but an approximately equivalent amount of enzyme was inhibited on each occasion (Table 1). Alternatively, for a fixed concentration of enzyme a decrease in the inhibitor concentration, from a concentration which normally gave complete inhibition to a half or one tenth of this value, gave levels of inhibition below those that might be expected for the proportions used.

These observations on the characteristics of the inhibition reaction may be explained in the following manner. Dibenamine hydrochloride solutions contain a reactive inhibiting species which is present and is stable in the acid media but is rapidly depleted in the near neutral media used here. The species is reactive towards a functional group on the enzyme which is only in the correct state of ionization for reaction

in near neutral media since the inhibition reaction did not occur to any appreciable extent in acid media. The reaction is rapid and consequently has the characteristics of a titration reaction between a functional group on the enzyme and a reactive species whose level is related to the dibenamine hydrochloride concentration.

It is considered by analogy with other β-halogeno-alkylamines that dibenamine exerts its alkylating action through the ethyleniminium ion, since the action of dibenamine in pharmacological systems may be prevented by a high concentration of thiosulphate ion, a reagent which reacts but slowly with the parent compounds (Nickerson & Goodman, 1948; Nickerson & Gump, 1949; Nickerson, Nomaguchi & Goodman, 1946). An attempt to show that the inhibitory species derived from dibenamine hydrochloride which participated in the reaction with α-chymotrypsin was the ethyleniminium ion was only partly successful. Addition of a high thiosulphate ion concentration to the acid solution of dibenamine hydrochloride before its use in the inhibition reaction substantially decreased the extent of the subsequent inhibition reaction but did not prevent it completely.

Dibenamine reacts via its ethyleniminium ion with the muscarinic receptors of rat isolated intestine (Beddoe, Nicholls & Smith, 1971) and decreases the response of the tissue to acetylcholine. Using this preparation we have shown in a qualitative manner that much higher concentrations of the ethyleniminium ion exist in an acid solution (Tyrode) of dibenamine than in a near neutral solution (Al Shabibi, Nicholls & Smith, to be published). These studies would support the view that the inhibitory species in the work described here is the ethyleniminium ion.

It is not possible to decide from the experiments described whether the noted effect of pH on the ethyleniminium ion concentration was due to a direct or indirect secondary effect of pH. The acidic and neutral media contained nucleophiles, e.g. Cl^-, HPO_4^{2-}, added to achieve the required pH, which differed in concentration and nucleophilic power. It would be expected that these adjuvents alone would control, to different extents, the ethyleniminium ion level in the two media.

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