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EVIDENCE FOR THE CARRIAGE OF SILVER BY SULPHADIMIDINE: INHIBITION OF PROTEOLYTIC ENZYMES

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1 Trypsin in free solution and trypsin-sepharose were shown to be inhibited by Ag^+ in the absence of Cl^-.

2 In the presence of Cl^- and absence of a suitable carrier, Ag^+ has no inhibitory action on trypsin or chymotrypsin.

3 Sulphadimidine bound Ag^+ in the presence of Cl^-, and carried the Ag^+ to both trypsin and chymotrypsin in free solution as well as to trypsin-sepharose leading to the inhibition of all these enzyme systems.

4 The neutral protease of tumour cell surfaces was inhibited by Ag^+ transported by sulphadimidine in the presence of Cl^-.

5 Kinetic data demonstrated the requirements for both Ag^+ and carrier to effect inhibition, the degree of inhibition being directly related to the molarity of each of these reagents.

6 The known inhibition of trypsin by Ag^+ binding to histidine in the active site has been defined in mechanistic terms employing the sulphonamide drug, sulphadimidine, to illustrate this exchange mechanism.

Introduction

The proteolytic enzymes trypsin and chymotrypsin have been demonstrated to be inhibited by silver ions (Steven, Podrazký, Al-Habib & Griffin, 1979). The trypsin-like neutral protease of Ehrlich ascites tumour cell surfaces has been shown to be inhibited by gold and zinc, both of which may be exchanged in reversible reactions leading to recovery of the enzymic activity (Short, Steven, Griffin & Itzhaki, 1981). The sperm-bound acrosin has also been shown to be inhibited by silver and zinc ions in a reversible manner (Steven & Chantler, 1981). Martinek, Savin & Berezin (1971) showed that Ag^+ reacted with histidine in the active centre of trypsin. Silver ions precipitate in the presence of Cl^- and thus free silver ions would not be expected to be capable of influencing physiological reactions. We have demonstrated that silver may be carried under physiological conditions as a complex with sulphadimidine (Ballinger, Brown, Griffin & Steven, 1982). In this study we have employed the carrier principle, elucidated with the haemolysis of erythrocytes, to demonstrate the carriage of Ag^+ by sulphadimidine leading to the inhibition of trypsin, trypsin-sepharose, the trypsin-like neutral protease of Ehrlich ascites tumour cells and chymotrypsin all in the presence of Cl^-. The present study extends the scope of carrier-mediated exchange reactions in the modifications of proteolytic activity by a drug, first shown for sodium aurothiomalate (Griffin & Steven, 1982). These results suggest that drugs may well have an influence on the enzymes of metabolic pathways *in vivo* which was not considered when the design of the drug was first conceived.

For a practical demonstration of a carrier mechanism, it is necessary to show that both the potential inhibitor (in this case Ag^+) and the carrier (sulphadimidine) play a controlling role in enzyme inhibition. This may be achieved by kinetic analysis employing the principle of incremental analysis in which the concentrations of all reagents are constant apart from one which controls the rate of product formation. In our example, Ag^+ alone has no inhibitory action in the presence of Cl^- unless the carrier is added, and the carrier itself has no direct action on the enzymes under study in this paper.

Methods

Direct β-naphthylamidase assay of trypsin-like enzymes in the presence of Ag^+ was carried out by modifying the fluorimetric analysis procedure of MacDonald, Ellis & Reilly (1966). We replaced the Tris-HCl buffer with 2% $NaHCO_3$, pH 8.0. The assay consisted of reacting the enzyme with 1.3 mg α-N-benzoyl-DL-arginine β-naphthylamide (BANA) in 3 ml 2% $NaHCO_3$ pH 8.0 for 1 h at 37°C followed by fluorimetric analysis of the product, β-naphthylamine. β-Naphthylamine is a carcinogen

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and, as such, we employed suitable precautions when employing BANA assays. We used disposable plastic gloves when dealing with BANA-containing solutions. The products of the enzymic digestions were collected in a large plastic bottle for safe disposal of the β-naphthylamine. Bulked waste products were made alkaline to pH 12 with NaOH and extracted with ether. The ether extract containing β-naphthylamine was adsorbed on oil and water-absorbent pellets and then incinerated.

The BANA assay employed the fluorescent emission at 410 nm produced when the product, β-naphthylamine, was excited at 335 nm. The presence of sulphadimidine quenched the fluorescence of β-naphthylamine and made the direct fluorimetric assay impossible. We therefore used the indirect assay, i.e. we employed sepharose-bound trypsin and cell-bound trypsin-like neutral protease which could be washed free of sulphadimidine before assay of β-naphthylamidase activity in 0.2 M Tris-HCl buffer pH 8.0. The problem of AgCl precipitation was avoided since all the excess Ag^+ had been removed in the washing with Tris-HCl before BANA analysis. We carried out the initial interaction of Ag^+, carrier, NaCl and particulate enzyme in 2 ml plastic centrifuge tubes. The excess reagents were removed by centrifugation, and the particulate enzyme washed three times with 1 ml 0.2 M Tris-HCl pH 8.0 buffer. The final stage was carried out by transferring the particulate enzyme to fresh 3 ml plastic tubes for β-naphthylamidase assay in 0.2 M Tris-HCl buffer (MacDonald *et al.*, 1966).

Fluorescein-labelled collagen fibrils were employed to assay trypsin proteolytic activity (Steven, Torre-Blanco & Hunter, 1975). In this assay, fluorescein-labelled peptides are solubilized from the telopeptide regions of the insoluble substrate. We used 7 mg substrate per tube and incubated for 1 h at 37°C in 3 ml 0.2 M Tris-HCl pH 8.0 buffer.

Fluorescein-labelled casein was employed to assay chymotrypsin activity (Steven, Griffin & Smith, 1981). The trichloroacetic acid-soluble peptides produced in this digestion were assayed fluorimetrically as described (Steven *et al.*, 1975).

Materials

Crystalline trypsin was obtained from Boehringer Mannheim Gmbh, West Germany, and crystalline α-chymotrypsin from Sigma Chemical Company, St. Louis, Mo. U.S.A. Ehrlich ascites tumour cells were grown intraperitoneally in mice (Itzhaki, 1972), the cells were washed in isotonic saline and suspended in saline (approximately 6×10^7 cells/ml) before use. Trypsin-sepharose was prepared according to the instructions of Pharmacia, Uppsala, Sweden, from activated sepharose-4B.

α-N-benzoyl-DL-arginine β-naphthylamide (BANA) was purchased from Sigma Chemical Company, St. Louis, Mo. U.S.A. Fluorescein-labelled polymeric collagen fibrils and fluorescein-labelled casein were prepared with fluorescein-isothiocyanate obtained from BDH, Poole. Sodium sulphadimidine was obtained from I.C.I. Pharmaceuticals Division, Macclesfield. Water and oil-absorbent granules were obtained from H.B. Hurd, Clarendon Road, Hyde.

Results and Discussion

In each series of experiments the initial enzymic activity is presented as 100% at A (Figure 1).

Inhibition of trypsin and trypsin-sepharose by Ag^+ in the absence of Cl^-

It was necessary to demonstrate the direct action of Ag^+ on trypsin and bound trypsin in the absence of Cl^- and therefore with no requirement for the sulphadimidine carrier. The data in Figure 1 show the direct action of incremental additions of $AgNO_3$ to trypsin (line AF) and trypsin-sepharose (curve AE) in the absence of Cl^- assayed with BANA in $NaHCO_3$ buffer. Silver readily inhibits both forms of trypsin although free trypsin is more readily inhibited than trypsin-sepharose. It will be shown that sulphadimidine interferes with the direct BANA assay and it was necessary to employ membrane-bound trypsin to overcome this difficulty.

Inhibition of trypsin by incremental additions of Ag^+ in Cl^- employing sulphadimidine as carrier and fluorescein-labelled polymeric collagen as substrate

In this reaction 55 mM sulphadimidine was mixed with incremental additions of $AgNO_3$ (0–150 μM final concentration) and then 77 mM NaCl added prior to 2 μg of trypsin. The proteolytic activity of the treated trypsin was then assayed with fluorescein-labelled polymeric collagen fibrils as substrate (Figure 1, curve AD). The data demonstrate that Ag^+ in the presence of Cl^- is carried to the active centre of trypsin by a fixed quantity of sulphadimidine resulting in progressive inhibition of the trypsin proteolytic activity. In the absence of sulphadimidine but with Cl^- no inhibition of trypsin was observed.

Inhibition of chymotrypsin by incremental additions of Ag^+ in Cl^- employing sulphadimidine as carrier and fluorescein-labelled casein as substrate

The data in Figure 1, curve AC, were obtained with chymotrypsin digestion of fluorescein-labelled casein

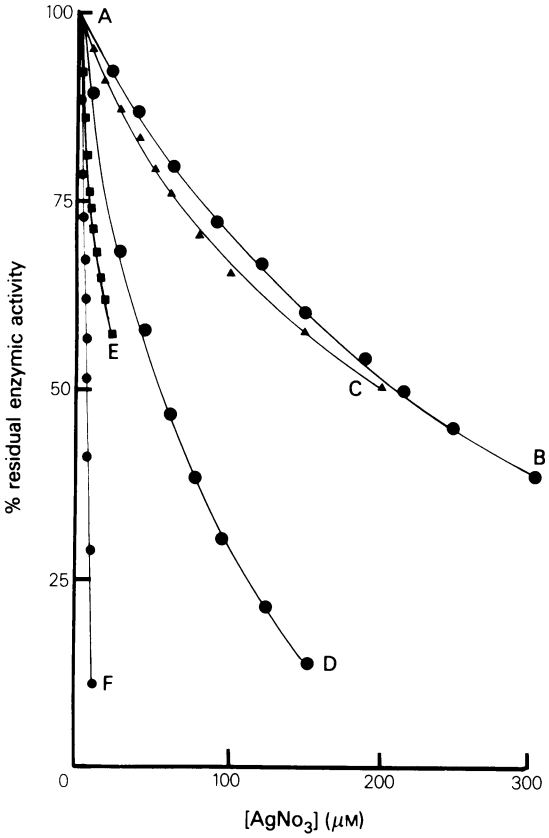

Figure 1 Inhibition of trypsin and chymotrypsin by Ag^+ in the absence of and in the presence of Cl^-. Point A represents the control enzymic activity in the absence of inhibitor and is presented as 100% on the vertical axis. See text for experimental details. Line AF represents the direct β-naphthylamidase assay of trypsin in the absence of Cl^-, whilst curve AE represents a similar analysis of trypsin-sepharose. Curve AD represents the inhibition of trypsin by Ag^+ in NaCl, employing sulphadimidine as carrier and fluorescence-labelled polymeric collagen as substrate. Curve AC represents the inhibition of chymotrypsin in the presence of Cl^-, assayed with fluorescence-labelled casein as substrate. Curve AB represents the inhibition of tumour cell surface neutral protease in Cl^-, employing 3×10^7 cells/tube and followed by the indirect BANA assay.

and are similar to the data in Figure 1, curve AD, obtained with trypsin. The object of this experiment was to demonstrate that silver carriage by sulphadimidine may result in inhibition of other proteolytic enzymes than trypsin. The choice of fluorescein-labelled casein as substrate was made on the basis that chymotrypsin cleaves casein but not the telopeptide regions of F-labelled collagen.

Inhibition of trypsin-sepharose by incremental additions of Ag^+ in Cl^- employing sulphadimidine as carrier and BANA as substrate

It was not possible to study the Ag^+ inhibition of free trypsin mediated by sulphadimidine and assayed with BANA since the carrier caused marked quenching of the fluorescent product, β-naphthylamine. This problem could be partly overcome by using an indirect assay based on insoluble trypsin-sepharose, from which the sulphadimidine was removed by washing before β-naphthylamidase assay. Under these conditions the quenching problem was by-passed, but it introduced a further complication that bound Ag^+ could be removed from the enzyme during the washing procedure employing Tris-HCl buffer. As a result we cannot strictly compare the data obtained from different experiments employing β-naphthylamidase activity, however the principle of carrier-mediated Ag^+ inhibition can be clearly demonstrated. The data are not presented here but the inhibition curve was similar to curve AB in Figure 1.

Inhibition of trypsin-sepharose by incremental additions of sulphadimidine to a fixed quantity of Ag^+ in Cl^- and BANA as substrate

In this series of experiments the Ag^+ concentration in all the tubes was the same (initially 100 μM) and the concentration of sulphadimidine added to each tube was varied (0–100 mM). It was observed that with no sulphadimidine no inhibition of trypsin-sepharose took place, but with increasing concentrations of sulphadimidine the bound enzyme became progressively more inhibited (data not shown, since Figure 2 shows a similar relationship employing the neutral protease attached to tumour cell surfaces).

Inhibition of tumour cell surface neutral protease by incremental additions of Ag^+ in Cl^- employing sulphadimidine as carrier and BANA as substrate

The β-naphthylamidase activity of the tumour cell surface neutral protease was progressively inhibited by incremental additions of Ag^+ to a fixed concentration of sulphadimidine in the presence of NaCl (Figure 1, curve AB). The initial concentration of added silver is again presented, as in the above experiment with trypsin-sepharose, although these concentrations were at least three times the concentration of Ag^+ in the BANA assay stage of the reaction (even if we assume that no silver was removed by washing). Clearly Ag^+ was carried by sulphadimidine, in the presence of Cl^-, to the cell surface and caused inhibition of the trypsin-like neutral protease located there. The data in Figure 1 curve AB demonstrate the validity of this indirect BANA assay. The quenching

by residual carrier could not provide this curve, since the amount of carrier initially added was the same in each tube, yet the tubes showed increased inhibition of neutral protease activity with increasing Ag^+ addition.

Inhibition of tumour cell surface neutral protease by incremental additions of sulphadimidine to a fixed quantity of Ag^+ in Cl^- and BANA as substrate.

The data presented in Figure 2 are similar to those in Figure 1 curve AB, yet in this case the initial concentration of Ag^+ remained constant whilst the only change was the initial concentration of added sulphadimidine. Three significant facts can be deduced from Figure 2. Firstly, when $AgNO_3$ is added with no carrier, no inhibition of the cell surface neutral protease took place. Secondly, the degree of inhibition was related to the amount of carrier added to the fixed quantity of Ag^+, i.e. the carrier played an essential role in Ag^+ transfer. Thirdly, when sulphadimidine alone was used with no Ag^+, then the carrier had no inhibitory action on the trypsin-like neutral protease.

In conclusion, we may say that sulphadimidine acts as a carrier of Ag^+ under physiological conditions leading to the inhibition of proteolytic enzymes in free solution and bound to the surfaces of sepharose and tumour cells (Figure 3). In Figure 3, reaction (1) indicates the interaction of Ag^+ with Cl^- to form an insoluble AgCl precipitate. If a carrier for silver is introduced before adding Cl^-, in our case sodium sulphadimidine, reaction (2) takes place with the formation of soluble silver sulphadimidine which retains the metal in the presence of added Cl^-. Silver may then be transferred from the carrier to the active centre of the enzyme in reaction (3) leading to enzymic inhibition. In the process the carrier is regenerated in reaction (4). It may well be found that many drugs act as carriers for metal ions which may modify enzymic action *in vivo*. It would seem that the carrier function of drugs in this role has yet to be explored fully. These two studies should help to illustrate possible techniques for demonstrating the principle of carrier mechanisms and exchange of metal ions.

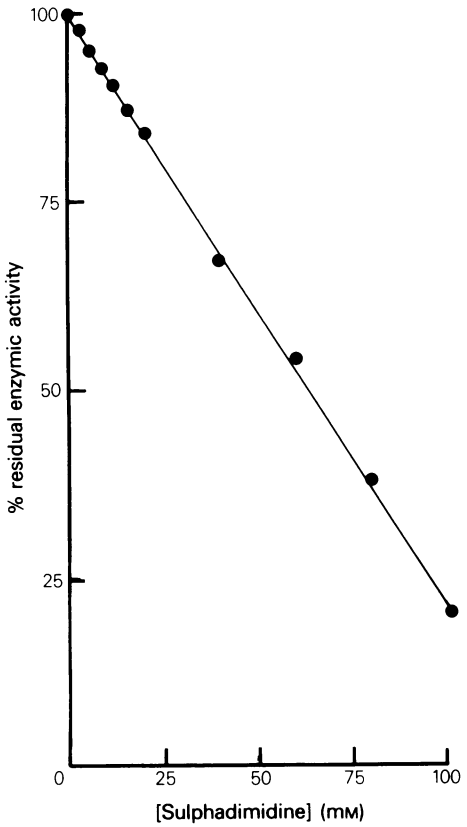

Figure 2 Inhibition of tumour cell surface neutral protease by incremental additions of sulphadimidine to a fixed quantity of Ag^+ in Cl^- and BANA as substrate. The conditions were the same as those in Figure 1, curve AB, except that we used 300 mM $AgNO_3$ followed by incremental additions of sulphadimidine before addition of NaCl and tumour cells.

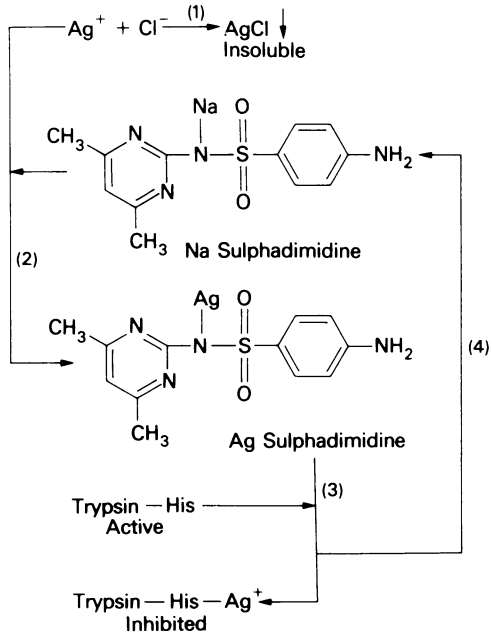

Figure 3 Proposed mechanism of silver carriage by sulphadimidine leading to enzymic inhibition.

General considerations

Many enzymes contain a reactive thiol in their active centres, for example the enzymes concerned with fatty acid metabolism. Thiols readily bind heavy metals and these enzymes would probably exhibit drug-mediated inhibition in the same manner as that described above.

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