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SHORT COMMUNICATIONS

A pepstatin–dextran conjugate as an inhibitor of proteinase-free human renin

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Pepstatins are a group of compounds, isolated from Streptomyces, that inhibit the activity of the so-called acid or, more recently renamed, aspartic acid active site proteinases, such as pepsin, cathepsin D and renin. Among pepstatins, pepstatin A (isovaleryl-L-valyl-L-valyl-4-amino-3-hydroxy-6-methylheptanoyl-L-alanyl-4-amino-3-hydroxy-6-methylheptanoic acid) is the most potent renin inhibitor. The effect of this peptide on the production of angiotensin I by inhibition of renin, both *in vivo* and *in vitro*, has been described [1–5].

The water solubility of pepstatin is too low, and its duration of action in *in vivo* studies too short, to be of much potential clinical or, for some purposes, experimental usefulness. In an attempt to overcome these problems, we have prepared a pepstatin–dextran conjugate (PDC) by coupling pepstatin A through its carboxyl terminal, which seems not to be essential for the effectiveness of the peptide, to high molecular weight dextran, a non-toxic and relatively non-allergenic polysaccharide that has long been used as a plasma expander in humans.

A preliminary report on the characteristics of this conjugate and its inhibition of impure human renin and of pepsin was published previously [6]. Since then we have found that impure preparations of human renin are heavily contaminated with proteinase activity [7]. This realization has prompted a re-examination of the kinetics of inhibition of proteinase-free renin,* which had previously been studied only with the impure human enzyme [8]. The present report presents details of the preparation and properties of PDC and describes its inhibition of proteinase-free human renin.

Materials and Methods

Cyanogen bromide was purchased from Eastman Organic Chemicals (Rochester, NY). Dextran (T-40) was from Pharmacia Fine Chemicals Inc. (Piscataway, NJ); this material has an average molecular weight of 40,000. 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide was from the Pierce Chemical Co. (Rockford, IL). [^{125}I]Angiotensin I was obtained from the New England Nuclear Corp. (Boston, MA). Dr. H. Umezawa (Institute of Microbial Chemistry, Tokyo, Japan) provided the pepstatin. All other materials used in this study were similar to those described previously [8].

Preparation of dextran–ethylene diamine conjugate. To 200 ml of a stirred solution of dextran in water (1%, w/v) adjusted to pH 10.7 with 0.5 N NaOH, cyanogen bromide (0.5 g) was added, followed by a second addition 30 min later. The pH was maintained at 10.7 during this process by addition of 0.5 N NaOH. Thirty minutes after the second addition of cyanogen bromide, the pH was adjusted to 9.0 by addition of 0.1 N HCl. After dialysis at 4° for 2 hr against 4 liters of 3 mM sodium carbonate (pH 9.0), ethylene diamine (0.5 g) was added. Coupling of ethylene diamine to cyanogen bromide activated dextran was then allowed to proceed for 12 hr ar 4°. An essential feature of this scheme of preparation is the relatively low concentration of cyanogen bromide used, which avoids irreversible precipitation of the complex during activation.

Preparation of the pepstatin–dextran conjugate (PDC). Pepstatin (15 mg) was coupled to 1 g of the dextran–ethylene diamine conjugate in 10 ml of a 1:1 mixture of pyridine and 0.1 M sodium phosphate buffer (pH 5.5) using 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide as the catalyst at room temperature for 12 hr. In this solvent mixture all of the reactants are soluble. After this period, the reaction mixture was dialyzed for 2 hr at room temperature against distilled water, 2 g of glycine was added, and the mixture was allowed to stand for another 12 hr at 4°. The solution was then dialyzed for 24 hr at 4°, after which the product was isolated by lyophilization. The addition of an excess of glycine following the coupling reaction is essential to saturate unreacted imidocarbonate groups and thus prevent the soluble conjugate from becoming insoluble during its isolation by freeze-drying.

Assays of renin. Enzyme assays, unless otherwise stated, were performed by the method of Bath and Gregerman [9] as later modified [5]. In this assay the substrate is a synthetic ^{125}I-labeled polymeric tetradecapeptide. In a few studies the reaction of renin with protein renin substrate was studied by the method of Haber *et al.* [10] in which generated angiotensin I is measured by radioimmunoassay.

Preparation of proteinase-free human renin. The purification of human renin was accomplished by affinity chromatography of crude human renin on hemoglobin-Sepharose-4B [11]. The kinetics of inhibition of this material are to be described in detail elsewhere.*

Kinetic studies. Inhibition of proteinase-free human renin by PDC was studied by the method of Ackerman and Potter [12] by varying the renin concentration in the absence (control) and presence of three concentrations of the inhibitor. Reaction velocity versus renin concentration was plotted graphically for each situation.

Results

Pepstatin–dextran conjugate. Amino acid analysis after acid hydrolysis of PDC indicated that 1 mg of the synthetic compound contained 20 μg of pepstatin. In control experiments in which no carbodiimide was added to the reaction mixture, no pepstatin was found, indicating that the peptide was not merely absorbed by dextran during preparation of the conjugate.

PDC had, within the range tested, the same solubility as the unmodified dextran, i.e. at least 100 mg/ml. A solution of PDC at this concentration would contain 2 mg/ml as pepstatin, in contrast to a water solubility of 20 μg/ml for the uncoupled peptide; solubility is thus increased at least 100-fold.

Inhibition of proteinase-free human renin by PDC. The kinetics of the inhibition of human renin at five different concentrations against three concentrations of the pepstatin–dextran conjugate are shown in Fig. 1. A plot

* G. Pourmotabbed, H. J. Chou and R. I. Gregerman, unpublished data.

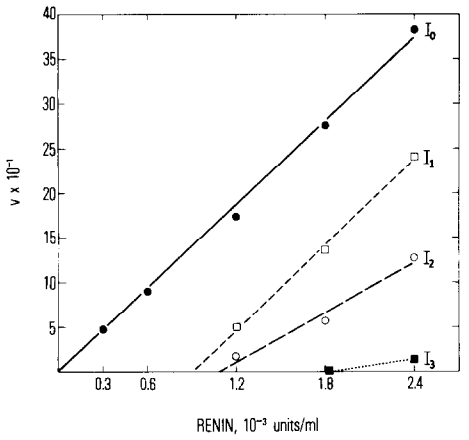

Fig. 1. Ackermann–Potter plot showing inhibition of proteinase-free human renin by dextran–pepstatin conjugate. The inhibitor lines that intersect the X-axis to the right of the origin represent the tight-binding (irreversible or pseudo-irreversible) character of the reaction. The reaction velocity, V, is in counts per minute generated per minute of incubation time. The concentration of substrate was 0.88 nM in the absence (●) and presence of inhibitor. Pepstatin–dextran conjugate concentrations were I_1, 1.45×10^{-10} M (□), I_2, 2.90×10^{-10} M (○), and I_3, 5.80×10^{-10} M (■) as pepstatin content.

of reaction velocity versus renin concentration (Ackermann–Potter plot) in the absence and presence of inhibitor yielded a series of lines that did not intersect at the origin, but, rather, intercepted the enzyme axis. The graphical representation corresponds to tight-binding (irreversible or pseudo-irreversible) inhibition. The inhibition by pepstatin differed from that described earlier for human renin where reversible inhibition was seen [5]. The difference was due to the presence of contaminating proteinases in the enzyme used in the earlier work [7,*]. The kinetics of inhibition of proteinase-free human renin were, in fact, similar for unconjugated pepstatin and PDC. With the labeled polymeric substrate assay used in these experiments, inhibition of renin was evident at concentrations of PDC in the range of 10^{-10} M (as pepstatin). Several additional experiments with different concentrations of enzyme, substrate, and inhibitor (pepstatin and PDC) were performed with essentially the same results.

Inhibition of the reaction of renin and protein renin substrate by PDC. The inhibition of angiotensin I production by human renin, acting on partially purified human and porcine renin substrate, was tested by incubation at pH 7.4 followed by immunoassay of angiotensin I. The concentration (in terms of pepstatin content) of PDC required to produce 50 per cent inhibition of 1×10^{-3} Goldblatt unit/ml of renin were 2.4×10^{-5} M for human renin substrate and 2.2×10^{-5} M for porcine substrate. These figures are only slightly higher than with pepstatin itself, viz. 1.8 and 1.4×10^{-5} M respectively. Although these concentrations of pepstatin appear to be rather high, the amount of enzyme used was also high, being at least ten times that in plasma. Since the inhibition was, in essence, irreversible, the amount of inhibitor required depended, in part, on the amount of enzyme present. As shown in Fig. 1, using another assay, much greater potency of PDC was evident under different conditions.

Discussion

The coupling of biologically active compounds to polymers has recently been increasingly explored. Several years ago we devised a rapid assay for renin in which a synthetic tridecapeptide substrate was coupled to polyglutamic acid and subsequently labeled with ^{125}I [9]. This peptide conjugate was used to allow rapid and simple separation of the cleavage products of renin's action through the grossly altered solubility characteristics of the polymeric substrate, as compared to the unbound peptide substrate. For a variety of reasons, others have attached enzymes such as amylase, trypsin, and catalase to soluble dextran [13], analgesic drugs and anti-inflamatory agents have been coupled to starch [14], and naloxone has been coupled to hydrazine-substituted polysaccharide [15]. Dextran-coupled enzymes have properties that are not only modified *in vitro* but that also exhibit altered characteristics *in vivo*. Comparison of the circulating half-life of native bacterial α-amylase with that of its dextran conjugate in rats showed the rate of removal of modified enzyme to be much slower than that of native enzyme [16]; the survival time of dextran-coupled arginase and carboxypeptidase G was extended in a similar fashion [17]. In addition, the dextran–enzyme conjugates were more resistant than the corresponding native enzymes to proteolysis.

Experimental use of pepstatin has heretofore been limited by the very low water solubility of the peptide and its short duration of action, almost certainly due to its rapid clearance from the circulation. To circumvent both the problem of limitation of dosage due to the low solubility of pepstatin and the short duration of action of the peptide, we have coupled pepstatin through its C-terminal carboxyl to several high molecular weight, water-soluble polymers, since the C-terminal carboxyl seems not to be essential for the effectiveness of the peptide. The most promising of these conjugates seems to be the one we presently describe in which pepstatin is coupled to dextran. The solubility of the conjugate is more than 100 times that of free pepstatin, while its potent inhibitory effects toward renin and pepsin, reported earlier, are almost the same as those of the unconjugated peptide. Although we have not examined the effectiveness of this material *in vivo*, we have shown its effectiveness at neutral pH *in vitro*. It seems likely that this material would, *in vivo*, have a much longer duration of action than the unconjugated peptide, perhaps a duration of action approaching that of the dextran polymer itself in the circulation.

Kinetic data for inhibition of proteinase-free human renin were obtained. These studies indicate irreversible or pseudo-irreversible inhibition of the reaction, which is similar to that of pepstatin itself. The implication of this result is that the inhibition of renin *in vivo* can be expected to depend not only on the inhibitor concentration but also on that of the enzyme. This type of inhibition should be especially advantageous for the inhibition of the relatively low levels of enzyme that exist in the circulation.

In summary, pepstatin, a potent but rather insoluble inhibitor of aspartic acid proteinases of the pepsin type, was coupled through its C-terminal carboxyl to dextran by the cyanogen bromide activation procedure. Pepstatin–dextran conjugate retained the high water solubility of uncoupled dextran and the potent inhibitory effect of pepstatin toward renin. Kinetic studies of the inhibition of proteinase-free human renin by the conjugate indicated tight-binding inhibition of the reaction, similar to that seen with pepstatin itself. These data suggest that this or similar conjugates of pepstatin with high water solubility and predictably slow metabolic clearance may have potential for *in vivo* use.

* G. Pourmotabbed, H. J. Chou and R. I. Gregerman, unpublished data.

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Inhibition of γ-aminobutyric acid stimulated [^{3}H]diazepam binding by benzodiazepine receptor ligands

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The recent demonstration of specific high affinity receptor sites for the benzodiazepines in brain tissue [1, 2] has provided insights concerning the mechanism of action of these drugs. Furthermore, the high correlation between receptor binding affinity and the pharmacologic potency of the benzodiazepines [3] indicates that this receptor is involved in mediating the action of these drugs and provides a convenient means for screening new potential anxiolytic and anticonvulsant drugs. Recent studies have focused on the isolation and characterization of endogenous ligands [4] for the benzodiazepine receptor. Using inhibition of specific [^{3}H]diazepam binding to assay fractionated tissue extracts, a growing list of putative endogenous ligands for the benzodiazepine receptor has emerged which includes the purines [5–7], nicotinamide [8], γ-aminobutyric acid (GABA) modulin [9], β-carboline ethyl esters [10], and various other unidentified fractions [11, 12]. The purines and nicotinamide are the only putative ligand candidates which also display benzodiazepine-like neurophysiologic [8, 13] and pharmacologic [8, 14] effects, but their relatively low receptor binding potencies have raised questions concerning their physiologic relevance as ligands or modulators of the receptor *in vivo*.

A large body of evidence indicates that the benzodiazepines exert at least some of their effects via the inhibitory neurotransmitter GABA. The benzodiazepines potentiate GABA-mediated processes [15] and antagonize convulsions caused by GABA depletion [16]. *In vitro* studies have also shown that GABA stimulates [^{3}H]diazepam binding to extensively washed synaptosomal membranes [17, 18]. The neurophysiological and biochemical evidence, therefore, clearly indicates that the benzodiazepine and GABA receptors are tightly coupled, although the biochemical basis of this coupling remains largely unknown.

We now report that the benzodiazepines and several putative endogenous ligand candidates inhibit GABA-stimulated [^{3}H]diazepam binding at concentrations 2- to 5-fold lower than those required to inhibit basal, non-GABA-stimulated binding.

Synaptosomal membranes were prepared from fresh rat forebrain tissue (male Sprague–Dawley, 100–125 g) according to the procedure described previously [4]. The P_2 pellet was washed four times in 50 original tissue volumes of 50 mM Tris–Cl buffer, pH 7.3 (assay buffer). The final membrane suspension was frozen for at least 16 hr at −20°. Immediately before use, the membrane suspension was thawed, centrifuged at 30,000 *g* for 30 min and resuspended in 50 volumes of assay buffer. Protein determinations were performed as described by Lowry *et al.* [19].

The [^{3}H]diazepam receptor binding assay was performed as described previously [3, 6] with minor modifications. Each assay contained 0.1 to 0.2 mg of the extensively washed membrane protein, [^{3}H]diazepam (New England Nuclear Corp., Boston, MA, 80 Ci/mmole), and the indicated additions. The total assay volume was 0.5 ml and the final buffer concentration 50 mM (assay buffer). The incubations were terminated by vacuum filtration using Whatman GF-B filters and four 3 ml washes with ice-cold assay buffer. The filters were counted by liquid scintillation counting in Aquasol. Under these conditions approximately 5000 dpm per assay was obtained at 1.25 nM [^{3}H]diazepam. Non-specific binding was determined by the incorporation of 3 μM unlabeled diazepam (Hoffmann-LaRoche, Nutley, NJ) and routinely represented 5 per cent of the total bind-