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Water-Soluble Macromolecular Complexes of Kallikrein, Bradykinin and Inhibitors of Kallikrein and Kininase II

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Pig pancreatic kallikrein, aprotinin (Trasylol), SQ 21541, an angiotensin I converting enzyme or kininase II inhibitor, and bradykinin were each coupled covalently to soluble dextran (m.w. 500,000). Dextran had been activated either with cyanogen bromide or sodium meta-periodate. Of the reactants, 23 to 56% were bound to activated dextrans. The activities of the complexes were determined in vitro by spectrophotometric technique or radioimmunoassay and bioassay. Depending on the mode of coupling and the test employed soluble macromolecular complexes retained 6 to 92% of the in vitro activity of the native compound.

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20. Abstract

Bradykinin coupled covalently to dextran was inactivated slower by converting enzyme than was free bradykinin.

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Water-Soluble Macromolecular Complexes of Kallikrein, Bradykinin and Inhibitors of Kallikrein and Kininase II

C. E. Odya, Y. Levin, E. G. Erdös

INTRODUCTION

Although proteins and peptides coupled to insoluble materials (1), including kallikreins and their inhibitors (2,3,4) and Hageman factor (5), have been employed frequently in vitro they could not be used in vivo because they are insoluble. The potential therapeutic usefulness of active, soluble dextran complexes of biologically important substances makes it desirable to prepare and evaluate such conjugates. Therefore, we have prepared soluble dextran complexes of kallikrein, aprotinin (Trasylol), SQ 21541, a synthetic hexapeptide inhibitor of kininase II or angiotensin I converting enzyme (6), (peptidyl dipeptidase), and bradykinin.**

MATERIALS AND METHODS

The details of the materials and techniques used were given in our previous publication (7).

Coupling to Cyanogen Bromide Activated Dextran (CNBr-Dextran)

Dextran (500,000 m.w.) was activated with cyanogen bromide according to the method of Axen et al. (8).

Solutions of aprotinin, 1 mg/ml, kallikrein, 5 mg/ml, and bradykinin, 2.5 mg/ml were prepared in 0.1 M sodium phosphate buffer, pH 7.6. One ml of each of these solutions was allowed to react with 4 ml of the CNBr-dextran solution for 60 h at 5^{o}.

The separation of free aprotinin and kallikrein from that bound to dextran was achieved by filtration at 5^{o} and 40 PSI N_2 through an Amicon XM100A membrane. Bradykinin was removed from that coupled to CNBr-dextran by dialysis at 5^{o} against 0.1 M sodium phosphate buffer, pH 7.6, 1 M sodium chloride, and water respectively.

Coupling to Sodium meta-periodate Activated Dextran (SMP-Dextran)

Dextran (500,000 m.w.) was oxidized with sodium meta-periodate.

Solutions of aprotinin, 0.4 mg/ml, kallikrein, 2.0 mg/ml, bradykinin, 1 mg/ml and SQ 21541, 1 mg/ml, were prepared in 0.1 M sodium phosphate buffer, pH 7.6. Equal volumes of oxidized dextran and of compounds to be coupled were allowed to react for either 16 or 60 h at 5^{o}. The reactive groups remaining on the oxidized dextran at the end of the coupling reaction were reduced with borohydride. This step also converts to stable secondary amines, the unstable Schiff bases that are formed when the peptides and proteins react with oxidized dextran.

The uncoupled compounds were separated from those bound as described above for CNBr-dextran.

Analysis of the Dextran Complexes

The amount of protein or peptide coupled to dextran was determined by amino acid analysis with a Beckman Model 121 automatic amino acid analyzer. The amount bound was calculated from known amino acid composition excluding methionine and cysteine. When SMP-dextran was used arginine was also excluded from the calculations.

Assays

Kallikrein activity was measured by following the hydrolysis of 1×10^{-3}M benzoylarginine ethylester (BAEe) by the enzyme at room temperature in a Cary 118 recording spectrophotometer at λ=254 nm (7). Aprotinin activity was measured by determining its inhibition of the hydrolysis of BAEe by kallikrein. The inhibitor was pre-incubated with the enzyme for 10 min at room temperature. SQ 21541 activity was measured by assaying its inhibition of the hydrolysis of hippuryl-glycyl-glycine (Hip-Gly-Gly;10^{-3}M) by purified swine kidney angiotensin

I converting enzyme (6).

The biological potencies of bradykinin-dextran complexes were determined on isolated guinea pig ileum and rat uterus preparations (7). The immunological activities of the complexes were assessed in a bradykinin radioimmunoassay that employed ^{125}I-Tyr^{5}-bradykinin as the labeled antigen, rabbit antibradykinin serum, and dextran coated charcoal to separate bound from free antigen (9). The bradykinin-dextran complexes were compared to bradykinin as inhibitors of labeled antigen binding.

The hydrolysis of bradykinin or bradykinin-dextran by purified swine kidney converting enzyme was determined in two ways. Bradykinin, 2 X 10^{-5}M, or an equivalent concentration of its dextran complex was incubated with the enzyme for varying lengths of time at 37^{o} and the residual bradykinin measured by radioimmunoassay. Free and dextran bound-bradykinin were also tested as inhibitors of the hydrolysis of Hip-Gly-Gly by converting enzyme (6). Purified swine kidney converting enzyme was equilibrated at 37^{o} for 10 min, then Hip-Gly-Gly (10^{-3}M) was added followed immediately by bradykinin or bradykinin SMP-dextran.

RESULTS

Kallikrein

From the amount of kallikrein allowed to react with CNBr-dextran, 56% had been coupled. Bound kallikrein had 72% of the esterase activity of the native enzyme (Fig. 1; Table 1). The activity of uncoupled kallikrein was 121 U BAEe per mg protein. (One U of enzyme activity equals 1 umole of BAEe cleaved in 1 min.)

Aprotinin (Trasylol)

Thirty-five percent of the aprotinin allowed to react with CNBr-dextran was coupled covalently (4,4 µg/mg). Fig. 2 shows the inhibition of BAEe esterase

activity of purified, uncoupled kallikrein by free and bound aprotinin. When the I_{50} values of the aprotinins were compared, the CNBr-dextran complex had 41% of the activity of the uncoupled inhibitor.(Table 1).

SQ 21541

The mean amount of SQ 21541 bound to SMP-dextran for 4 preparations was 9.1 µg per mg dextran (SD 0.8). Based on the total amount of SQ 21541 allowed to react in these preparations, 38% (SD 4.4) had been bound. SQ 21541 SMP-dextran was tested as an inhibitor of converting enzyme. Calculated from the I_{50} values of the 4 preparations. SQ 21541 bound to SMP-dextran has 24% (SD 4.0) the inhibitory activity of the uncoupled peptide.(Table 1).

Bradykinin

The amount of bradykinin bound to SMP-dextran in 5 preparations was 10.2 µg per mg dextran (SD 2.7). Of the total amount of bradykinin allowed to react with dextran, 45% (SD 15) had been bound. The corresponding figures for bradykinin coupled to CNBr-dextran were 7 µg per mg dextran and 23%.(Table 1).

The relative biological potency (unbound bradykinin = 100%) of bradykinin SMP-dextran was 29% (SD 12) when assayed on the isolated rat uterus and 18% (SD 8) when assayed on the isolated guinea pig ileum. The bradykinin CNBr-dextran complex had only 6% (SD 6) the biological potency of bradykinin on the rat uterus.

The relative immunological activities of the bradykinin dextran complexes were determined by radioimmunoassay. Estimates of the relative immunological activities (bradykinin = 100%) were made by comparing the concentrations that yielded 50% inhibition of the binding of $^{125}I\text{-}Tyr^{5}$ bradykinin to the antiserum. The relative immunological activity of the bradykinin SMP-dextran was 80% (SD 17) while that of bradykinin CNBr-dextran was 92% (SD 30).

The hydrolysis of bradykinin and bradykinin bound to SMP-dextran during

incubation at 37° with purified swine kidney converting enzyme was followed by measuring the residual immunoreactive bradykinin in a radioimmunoassay.

Bradykinin coupled to SMP-dextran was hydrolyzed much slower by kininase II than uncoupled bradykinin. At the end of 1 h of incubation, about 80% of the kinin SMP-dextran conjugate was still immunologically active while native bradykinin was completely inactivated presumably by the removal of C-terminal dipeptide Phe-Arg (6).

The effects of bradykinin and bradykinin SMP-dextran on the rate of hydrolysis of Hip-Gly-Gly by converting enzyme were also studied. Bradykinin bound to SMP-dextran was less effective than native bradykinin in inhibiting the hydrolysis of Hip-Gly-Gly. For the same decrease in the rate of Hip-Gly-Gly hydrolysis, coupled bradykinin had to be present in higher concentration than free bradykinin. Oxidized-reduced dextran itself inhibited the hydrolysis of Hip-Gly-Gly, although this was only about 20% of the inhibition obtained with comparable concentrations of the bradykinin SMP-dextran complex.

Non-covalent Binding

To establish whether the activity of the kallikrein, aprotinin, SQ 21541, and bradykinin dextran complexes was due to covalently bound material and not to material adsorbed on the dextrans, control preparations were made in which the protein and the peptides were incubated with either non-activated dextran or oxidized-reduced dextran and the mixtures carried through the various purification steps. None of the control preparations had activity that could be attributed to adsorbed material.

DISCUSSION

These experiments have shown that components of the kallikrein-kinin system covalently coupled to high m.w. soluble dextran retain activity in

in vitro. These include kallikrein, aprotinin, bradykinin, and the angiotensin I converting enzyme or kininase II inhibitor SQ 21541. They have biological importance and some are even used clinically. For example / SQ 20881, a nonapeptide which is structurally related to the hexapeptide SQ 21541, has been used in clinical experiments to lower the elevated blood pressure of hypertensives. This compound can block the conversion of angiotensin I to II or the inactivation of bradykinin by inhibiting converting enzyme or kininase II (6).

The coupling of kallikrein to CNBr-dextran did not appear to affect its esterase activity since plotting the activity against the enzyme concentration yielded parallel curves for free and bound enzyme (Fig. 1). The kallikrein CNBr-dextran complex was found to retain approximately three-fourths the esterase activity of uncoupled kallikrein. This compares favorably with kallikrein bound to the hydroxysuccinimide ester of polyvinylpyrrolidone which had about one-fourth the activity of free kallikrein on BAEe (10).

Bradykinin coupled to SMP- or CNBr-dextran was still biologically and immunologically active. The immunological activity of either complex was always greater than its biological activity. Possibly there is more steric hindrance to the accomodation of the macromolecular kinin complexes at receptor sites than there is at antibody binding sites.

Bradykinin SMP-dextran was less susceptible than native bradykinin to hydrolysis by purified swine kidney converting enzyme or by the enzyme in human vascular endothelial cells grown in tissue culture (11). The mechanism of this protection could be due to the increased size of the molecule. The bradykinin SMP-dextran complex inhibited Hip-Gly-Gly hydrolysis by converting enzyme but less than free bradykinin did. This also indicates that the dextran bound peptide has a lower affinity for the enzyme than free bradykinin. The decreased *in vitro*

inactivation of the bradykinin-dextran complex by converting enzyme suggests that this complex may have a longer lasting activity *in vivo* than free bradykinin.

In summary binding various components of the kallikrein-kinin system to dextran resulted in soluble macromolecular complexes with a high percent incorporation. The complexes retained significant amounts of activity *in vitro*.

SUMMARY

Pig pancreatic kallikrein, aprotinin (Trasylol), SQ 21541, an angiotensin I converting enzyme or kininase II inhibitor, and bradykinin were each coupled covalently to soluble dextran (m.w. 500,000). Dextran had been activated either with cyanogen bromide or sodium meta-periodate. Of the reactants, 23 to 56% were bound to activated dextrans. The activities of the complexes were determined *in vitro* by spectrophotometric technique or radioimmunoassay and bio-assay. Depending on the mode of coupling and the test employed the soluble macromolecular complexes retained 6 to 92% of the *in vitro* activity of the native compound. Bradykinin coupled covalently to dextran was inactivated slower by converting enzyme than was free bradykinin.

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Fig. 1. Effect of increasing the concentration of free and bound kallikrein on the rate of BAEe hydrolysis. O———O Purified pig pancreatic kallikrein. O———O Kallikrein bound to CNBr-dextran. Abscissa: Log concentration of kallikrein in µg per ml. Ordinate: Rate of hydrolysis of BAEe in $O.D._{254}$ per min. (From Odya et al. Biochem. Pharmacol. In press)

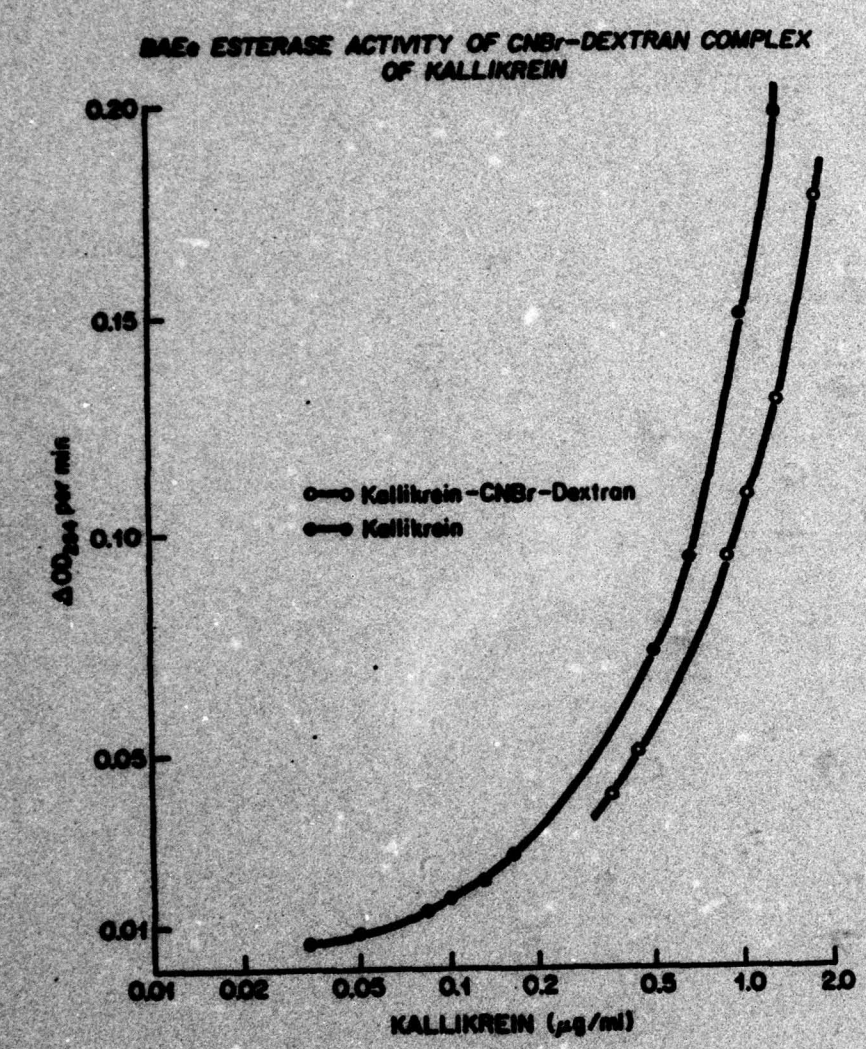
Fig. 2. Inhibition of the enzymatic hydrolysis of BAEe by free and bound aprotinin (Trasylol). Highly purified pig pancreatic kallikrein was the source of enzyme. O———O Aprotinin. O———O Aprotinin bound to CNBr-dextran. Abscissa: Log concentration of aprotinin in ng per ml. Ordinate: Percent inhibition. (From Odya et al. Biochem. Pharmacol. In press)

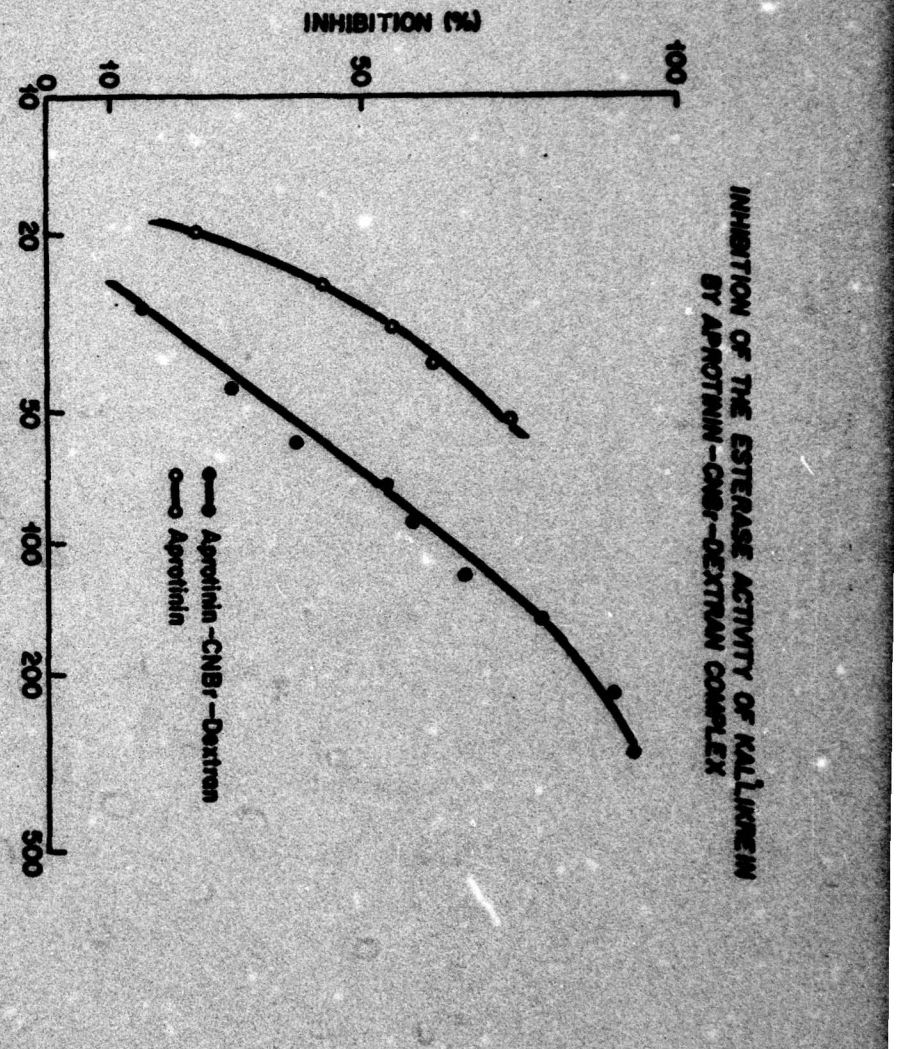
TABLE 1

Activities of Soluble Dextran Complexes *In Vitro*

Substances Coupled	Yield (%)	Test	Activity (Unbound = 100%)
Kallikrein[1]	56	Esterase	72
Aprotinin[1]	35	Kallikrein inhibition	41
SQ 21541[2]	38	Kininase II inhibition	24
Bradykinin[1,2]	23[1] 45[2]	Rat uterus	1/6 2/29
		Guinea pig ileum	18
		Kininase II inhibition	≈20
		Radioimmuno-assay	92 80
		Cleavage by kininase II	20

1. Cyanogen bromide activated dextran.
2. Sodium meta-periodate activated dextran.


BAEe ESTERASE ACTIVITY OF CNBr-DEXTRAN COMPLEX OF KALLIKREIN
0.20
0.15
0.10
0.05
0.01
ΔOD405 per min
Kallikrein-CNBr-Dextran
Kallikrein
0.01
0.02
0.05
0.1
0.2
0.5
1.0
2.0
KALLIKREIN (µg/ml)


INHIBITION OF THE ESTERASE ACTIVITY OF KALLIKREIN BY APROTININ-CNBr-DEXTRAN COMPLEX
INHIBITION (%)
0
10
50
100
APROTININ (ng/ml)
10
20
50
100
200
500
Aprotinin-CNBr-Dextran
Aprotinin