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BINDING OF LYSOZYME TO BRUSH BORDER MEMBRANES OF RAT KIDNEY

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The binding of ^{125}I-labelled egg-white lysozyme to isolated brush border membranes of rat kidney cortex was investigated. The lysozyme binding was reversible and saturable. The Scatchard plot revealed a one-component binding type with a dissociation constant of 7.8 μM and 15.6 nmol/mg membrane protein for the number of binding sites. The binding of the basic lysozyme could be reduced by basic amino acids such as L-lysine, L-ornithine or L-arginine, while neutral amino acids such as L-citrulline or L-alanine had no effect. The inhibitory effect of lysine was competitive.

Introduction

After glomerular filtration low molecular weight proteins are taken up from the renal proximal tubule by endocytosis [1–3]. Lysozyme (mol.wt. 14000) has been used as a test protein for uptake studies [4–6] and shown to be subsequently digested in lysosomes [7]. In clearance [8,9], whole kidney [10,11] and microperfusion studies [10], the tubular protein uptake was found to be inhibited by basic amino acids, but the mechanism of this inhibitory process is still unclear. Since earlier binding studies [12–14] suggested that the first step in the protein reabsorption process consists of an initial protein binding to brush border membranes, we investigated the interaction of ^{125}I-labelled egg-white lysozyme to isolated brush border membranes from rat kidney. The inhibitory effect of basic amino acids on lysozyme uptake could be localized to the first step in the process, the binding of the protein to the brush border membrane. Preliminary data have been published as an abstract [15].

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Methods

Isolation of brush border membrane fraction

For each experiment, 25 male Wistar rats (Winkelmann, Kirchborchen, F.R.G., each rat weighing 180–220 g) were killed by cervical dislocation; their kidneys were removed and placed in ice-cold buffer 1 (0.25 M sucrose/0.01 M triethanolamine-HCl, pH 7.4). All further procedures were done at 4°C. The kidneys were decapsulated and the kidney cortex was dissected with a razor blade, minced and then homogenized. Membrane fractionation [16] was done in buffer 1. The enrichment of brush border membrane compared to the starting homogenate was 4.95 ± 0.58 ($n = 11$)-times based upon the comparison of the specific alkaline phosphatase activity in both fractions. In four membrane preparations acid phosphatase, (Na^+-K^+)-ATPase and glucose-6-phosphatase were determined. The mean ratio of the specific activity found in the brush border membrane fraction compared to the specific activity of the enzymes measured in the starting homogenate amounted to 0.72 for acid phosphatase, 0.92 for (Na^+-K^+)-ATPase and 1.18 for glucose-6-phosphatase.

Quantitative protein and enzyme assay

Alkaline phosphatase (EC 3.1.3.1) was determined by the Merckotest method (Merck, Darmstadt, F.R.G.), in which *p*-nitrophenylphosphate is used as a substrate. Protein was determined [17] with bovine serum albumin as a standard. The determination of acid phosphatase (EC 3.1.3.2) was carried out with *p*-nitrophenylphosphate as substrate [18]. The activity of Mg^{2+}-ATPase and (Na^+-K^+)-ATPase (EC 3.6.1.3) was measured in 75 mM Tris-HCl buffer, pH 7.6, with 3 mM Tris-ATP as substrate [19]. Glucose-6-phosphatase (EC 3.1.3.9) was determined as described [20].

Binding studies

According to our previous binding studies [21–23] aliquots of 100 µl of each of the following solutions were pipetted into ice-cold (0°C) polyethylene tubes (Eppendorf Micro test tubes, Eppendorf Gerätebau, Hamburg, F.R.G.):

1. Buffer 1 3 × concentrated (0.75 M sucrose/0.03 M triethanolamine-HCl, pH 7.4).
2. Bovine serum albumin (1 mg/ml) in doubly-distilled water.
3. Inhibitor substance and/or D-[^{14}C]mannose in doubly-distilled water.
4. ^{125}I-labelled lysozyme in 0.145 M NaCl, pH 7.4.
5. Brush border membrane fraction resuspended in buffer 1.

After 2 min shaking in a rotary mixer, the tubes were centrifuged for 2 min at 12000 rev./min (Eppendorf Microsystem). In some experiments the supernatant was carefully separated from the pellet with a pipette and the entire supernatant sample (together with the pipette tip) as well as the pellet plus the tube was counted for radioactivity in a Packard Auto-Gamma scintillation spectrometer (model CP 153). If D-[^{14}C]mannose was used, an aliquot of the supernatant was counted for radioactivity in 10 ml Insta-Gel (Packard) and 1 ml doubly-distilled water. In these cases 100 µl solubilizer NCS® (Amersham-Buchler, Braunschweig, F.R.G.) was added to the pellet and after resuspension, the mixture was transferred quantitatively into a scintillation vial. All samples containing [^{14}C]mannose were first counted in a liquid scintillation system (Nuclear Chicago, Mark II) and then in the Gamma Spectrometer. In the liquid scintillation system double-labelling counting techniques were employed to simultaneously determine ^{125}I-labelled lysozyme and [^{14}C]mannose using ^{3}H/^{14}C discrimination. The recovery of ^{125}I and ^{14}C activities added was 97.6 ± 6.4% (n = 483) for ^{125}I-labelled lysozyme and 98.8 ± 5.6% (n = 533) for D-[^{14}C]mannose.

Test substances

All concentrations are given for 100 µl aliquots. Egg-white lysozyme (EC 3.2.1.17, Sigma, St. Louis, MO, U.S.A., grade I) was labelled with carrier-free ^{125}I-labelled iodine monochloride (Amersham) using a previously published method [24,25]. The ^{125}I-labelled lysozyme stock solution with a specific activity of 200 µCi/mg protein was diluted with isotonic sodium chloride (pH 7.4) to a lysozyme concentration of 8 µg/ml. The enzymatic activity of the labelled lysozyme was always more than 85% that of unlabelled lysozyme. For the following lysozyme concentrations the specific activity was changed by adding unlabelled egg-white lysozyme: 80 µg/ml, 800 µg/ml and 8000 µg/ml. Pilot experiments showed that the binding of lysozyme/mg membrane protein is essentially the same despite a 100-fold change in the specific activity of the lysozyme tested. The lysozyme binding to brush border membranes was reversible as shown in experiments with and without preincubation with unlabelled lysozyme. The binding studies were performed in the presence of bovine serum albumin (99%, Serva, Heidelberg, F.R.G.) in a concentration of 0.2 mg/ml which has no effect on lysozyme binding to isolated brush border membranes. The free iodine in ^{125}I-labelled lysozyme solutions were always less than 1% as determined via trichloracetic acid precipitation. The percentage value of free iodine did not increase during the binding studies under our experimental conditions. This was shown by comparing the percentage value of free iodine in the supernatant of incubations in the presence and absence of brush border membranes.

The following substances (Merck, Darmstadt, F.R.G.) were used as inhibitors: L-lysine-HCl (27, 137, 274, 547 and 1368 mM), L-ornithine-HCl, L-arginine-HCl, L-citrulline, L-alanine, all at 274 mM. The addition of the basic amino acids led to

a slight decrease of the pH values in the final incubation medium for binding studies. These slight changes in the pH values have no influence on lysozyme binding as shown in control experiments and in experiments with enhanced buffer concentration.

The brush border membrane fraction was resuspended in the isolation buffer to a protein concentration of 3.77 ± 0.79 mg/ml.

In the presence of brush border membranes there was no measurable lysozyme binding to the polyethylene tubes. As in our earlier studies [18,19] we calculated the dead space by using D-[^{14}C]mannose (spec. act. 0.29 mCi/mg, N.E.N., Boston, MA, U.S.A.) which was found not to bind to the brush border membranes. The dead space was determined to be 5%. The actual calculation was carried out on the basis of measurements of the dead space.

Results

The amount of bound lysozyme/mg membrane protein approached saturation with increasing free concentrations of lysozyme (Fig. 1). A Scatchard plot [26] revealed a single binding component type (Fig. 2) with a dissociation constant of 7.8 μM and of 15.6 nmol binding sites/mg membrane protein. Basic L-amino acids at a final concentration of 55 mM (lysine, arginine, ornithine) inhibited the ^{125}I-labelled lysozyme binding to the brush border membranes, while neutral L-amino acids (citrulline, alanine) in equimolar concentrations had no effect (Fig. 3). This inhibitory effect of lysine is dose-dependent (Fig. 4) and competitive (Fig. 5) as shown in the double-reciprocal plot [27].

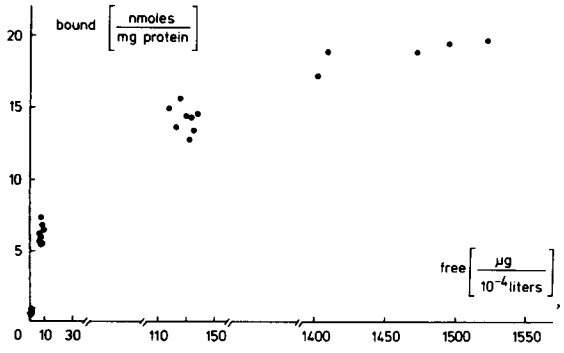

Fig. 1. Binding of ^{125}I-labelled lysozyme expressed as nmol lysozyme bound/mg membrane protein as a function of free concentration of lysozyme in the incubation medium. Note that the abscissa is twice interrupted. Each symbol represents the mean value of four separate observations.

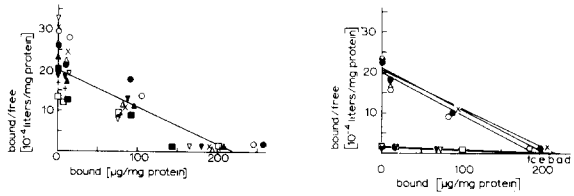

Fig. 2. Binding of ^{125}I-labelled lysozyme to brush border membranes (Scatchard plot). Each symbol represents the mean value of four separate observations. Different symbols are used for 10 separate experiments. The line represents the mean regression curve ($y = -0.0921x + 20.22$; $r^2 = 0.699$).

Fig. 3. Binding of ^{125}I-labelled lysozyme to brush border membranes (Scatchard plot) in the absence and presence of different L-amino acids at a final concentration of 55 mM. Each symbol represents the mean value of four separate observations.

a. ●———●, absence of amino acids
($y = -0.0952x + 20.90$; $r^2 = 0.951$)
b. ×———×, L-alanine
($y = -0.1021x + 21.11$; $r^2 = 0.966$)
c. +———+, L-citrulline
($y = -0.1041x + 20.09$; $r^2 = 0.916$)
d. ○———○, L-arginine
($y = -0.0077x + 1.52$; $r^2 = 0.347$)
e. △———△, L-ornithine
($y = -0.0070x + 1.57$; $r^2 = 0.618$)
f. ▲———▲, L-lysine
($y = -0.0084x + 1.59$; $r^2 = 0.823$)

Discussion

The cationic low molecular weight protein lysozyme is reabsorbed by proximal tubular cells via adsorptive endocytosis [4]. The first step in the chain of events has to be the binding of lysozyme to the luminal cell membrane. As shown in this study with an isolated membrane fraction this binding of lysozyme to brush border membranes is rapid, reversible, saturable and inhibited competitively by cationic amino acids. Thus, the lysozyme binding as the first step in the protein reabsorption shows characteristics of the lysozyme reabsorption. Renal lysozyme reabsorption in clearance

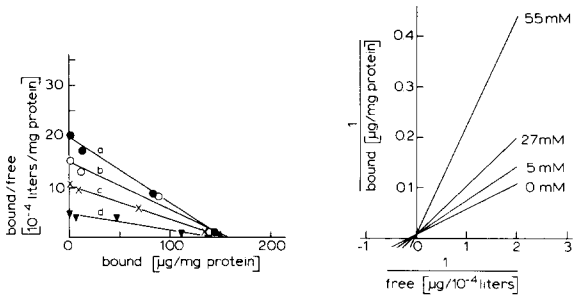

Fig. 4. Binding of ^{125}I-labelled lysozyme to brush border membranes (Scatchard plot) in the absence and presence of the following final concentrations of L-lysine. Each symbol represents the mean value of four separate observations.

a. ●———●, absence of L-lysine
($y = -0.1292x + 19.64$; $r^2 = 0.994$)
b. ○———○, 5 mM L-lysine
($y = -0.0948x + 14.97$; $r^2 = 0.971$)
c. ×———×, 27 mM L-lysine
($y = -0.0679x + 10.32$; $r^2 = 0.995$)
d. ▼———▼, 55 mM L-lysine
($y = -0.0329x + 4.63$; $r^2 = 0.949$)

Fig. 5. Double-reciprocal plot of ^{125}I-labelled lysozyme binding to brush border membranes at different L-lysine concentrations (data from Fig. 4) to show the competitive type of inhibition (only the intersection with parts of the regression lines are shown). Absence of L-lysine ($y = 0.0503x + 0.0079$; $r^2 = 0.999$), 5 mM L-lysine ($y = 0.0662x + 0.0104$; $r^2 = 0.999$), 27 mM L-lysine ($y = 0.0948x + 0.0087$; $r^2 = 0.999$), 55 mM L-lysine ($y = 0.2142x + 0.0102$; $r^2 = 0.999$).

studies [5] and the proximal tubular lysozyme reabsorption in microperfusion studies [4] is a saturable process. In the microperfusion studies [4] the intraluminal lysozyme concentration at which the tubular reabsorption rate is half-maximal (K_t value = 29 μM) is in the same range as the dissociation constant in this binding study. The inhibitory effect of basic amino acids on renal protein reabsorption was first proposed by the clinical observation, that after infusion of large doses of basic amino acids an elevated tubular proteinuria occurs [8,9]. Lysozyme reabsorption [10,11] and lysozyme binding to isolated brush border membranes in this study is strongly inhibited by cationic amino acids while neutral amino acids have no effect. The inhibitory effect of the cationic amino acid lysine on the binding of the cationic lysozyme is competitive. In addition lysozyme reabsorption [11] and the binding of the cationic peptide aprotinin to brush border membranes [13] was inhibited by several basic substances. These data clearly indicate that molecular charge is an important factor in the competition for common endocytic transport sites, but molecular parameters other than charge and size may also play a role [11]. Therefore, the binding as the initial event in the endocytic process is supposed to take place between positively-charged groups of the protein molecule and negative sites on the brush border membrane [9,11,13,28]. Selectivity of protein reabsorption could be determined by different affinities of basic substances to the binding site. In earlier studies [28] we have shown that renal endocytic vesicles are rich in negatively-charged phospholipids which might act as binding sites for positively-charged macromolecules. Indeed there is evidence that acidic phospholipids are an integral component of the renal brush border membrane binding site for the aminoglycoside antibiotics which are also endocytized by the kidney [29].

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