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EFFECT OF LOW-MOLECULAR-WEIGHT PROTEINS ON PROTEIN (LYSOZYME) BINDING TO ISOLATED BRUSH-BORDER MEMBRANES OF RAT KIDNEY

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Filtered proteins including the low-molecular-weight protein lysozyme are reabsorbed by the proximal tubule via adsorptive endocytosis. This process starts with binding of the protein to the brush-border membrane. The binding of ^{125}I-labelled egg-white lysozyme (EC 3.2.1.17) to isolated brush-border membranes of rat kidney and the effect of several low-molecular weight proteins on that binding was determined. The Scatchard plot revealed a one-component binding type with a dissociation constant of 5.3 μM and 53.0 nmol/mg membrane protein for the number of binding sites. The binding of the cationic lysozyme was inhibited competitively by the addition of cationic cytochrome *c* to the incubation medium, while the neutral myoglobin had no effect. The anionic β-lactoglobulin A inhibited the lysozyme binding in a noncompetitive manner. These data suggest that the binding takes place between positively charged groups of the protein molecule and negative sites on the brush-border membrane, and, the competition between the cationic cytochrome *c* and the cationic lysozyme for the binding sites may be responsible for the inhibitory effect of cytochrome *c* on renal lysozyme reabsorption. The binding step at the brush-border membrane appears to be cation-selective.

Introduction

Lysozyme as other low-molecular weight proteins is easily filtered in the kidney and taken up by proximal tubular cells from the ultrafiltrate via endocytosis [1–6]. From the endocytic vacuoles the protein is transferred to the lysosomes, where catabolism of the protein occurs. There is increasing evidence for competition between proteins during their renal reabsorption [6–11]. Clearance [2–4] and microperfusion studies [5] have shown that lysozyme reabsorption is a saturable process indicating that the cellular lysozyme uptake occurs by adsorptive endocytosis. Thus, the initial event in lysozyme reabsorption has to be the binding of lysozyme to the brush-border membrane. Recently, we characterized the lysozyme binding to isolated brush-border membranes of rat kidney [12].

In our previous microperfusion studies several cationic low-molecular weight proteins inhibited the proximal tubular reabsorption of the cationic lysozyme while at comparable concentrations the neutral myoglobin and the acidic β-lactoglobulin A had no effect [9,10]. These results indicated competition between cationic proteins for common transport sites. In the present study lysozyme binding to isolated brush-border membranes was determined to solve the question, if the inhibitory effect of cationic proteins on lysozyme reabsorption [9,10] may be caused by inhibition of lysozyme binding at brush-border membrane sites or by modification of subsequent steps in the endo-

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cytic process. Preliminary data have been published as an abstract [13].

Methods

Isolation of brush-border membrane fraction

For each membrane preparation 12 g of frozen renal cortical slices (stored in liquid nitrogen) were used. The dissected cortices were obtained from male Wistar rats (Winkelmann, Kirchborchen, F.R.G.). Rats (180–220 g) were anesthetized with an intraperitoneal injection of thiobutabarbital (Inactin®, Byk Gulden, Konstanz, F.R.G.), 80–100 mg/kg body weight. After opening of the abdominal cavity and tapping of the distal abdominal aorta with a 20 g needle the proximal aorta was clamped and the kidneys were perfused with approx. 15 ml of an ice-cold isotonic saline solution until cleared of blood. The kidneys were then excised, decapsulated, decorticised with a razor blade and the obtained tissue (approx. 0.4 g/kidney) was placed immediately in liquid nitrogen in sealed cryotubes (Nunc, Denmark) for further preparation. The actual membrane fractionation [14] was carried out in buffer 1 (50 mM mannitol/2 mM Tris-HCl buffer, pH 7.0). After gentle thawing in a circulating water bath (37°C) the minced cortices were transferred into 30 vol. (w/v) of ice-cold buffer 1 and then homogenized. After adjusting the calcium concentration to a final concentration of 10 mM and stirring in an ice bath for 10 min, the homogenate was centrifuged at 3000 × *g* for 15 min (Sorvall RC-5 refrigerated centrifuge). The cautiously decanted supernatant was centrifuged again at 43000 × *g* for 20 min and the obtained pellet was resuspended in buffer 1 using a 1 ml syringe and a 23 g needle. Again the material was washed at 43000 × *g* for 20 min and the final pellet was resuspended for further experiments in buffer 2 (0.25 M sucrose/0.01 M triethanolamine-HCl, pH 7.4).

To evaluate the quality of the brush-border membrane preparations the activities of the following enzymes were used as markers, neutral α-glucosidase and alkaline phosphatase for the brush-border membrane, β-glucuronidase for lysosomes and $(Na^+ + K^+)$-ATPase for basolateral plasma membranes. The mean ratio ($n = 10$) of the specific activity found in the brush-border membrane fraction compared to the specific activity of the enzymes determined in the starting homogenate amounted to 12.9 for neutral α-glucosidase, 8.1 for alkaline phosphatase, 0.52 for β-glucuronidase and 0.39 for $(Na^+ + K^+)$-ATPase. As indicated by these enrichment factors of the brush-border membrane enzymes the purification of the brush-border membrane is approx. 10-fold. The specific activities of the non-brush-border enzymes were reduced in the brush-border membrane preparations, indicating low contamination by lysosomes and basolateral plasma membranes. The total amount of isolated membrane protein for each membrane preparation reached 54.3 ± 7.1 mg ($n = 10$) representing 1.8% of the membrane protein found in the homogenate.

Aliquots of 500 µl brush-border membrane fraction were sealed in cryotubes and placed in liquid nitrogen for further experiments. In some experiments freshly isolated brush-border membranes were used. No difference was observed in lysozyme binding between frozen and freshly isolated brush-border membranes during our experiments, when the maximum immersion period of membranes in liquid nitrogen did not exceed 21 days.

Quantitative protein and enzyme assay

Protein was determined with bovine serum albumin as a standard [15] and neutral α-glucosidase (EC 3.2.1.20) with maltose as substrate as described recently [16,17].

Alkaline phosphatase (EC 3.1.3.1) was assayed by the Merckotest method (Merck, Darmstadt, F.R.G.), in which *p*-nitrophenylphosphate is used as a substrate. β-Glucuronidase (EC 3.2.1.31) was determined by using the Diagnostic Kit β-Glucuronidase (Sigma, St. Louis, MO, U.S.A.) with phenolphthalein glucuronic acid as substrate. 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 [18].

Binding studies

The technique used was similar to our previous binding studies [12,19–21]. All concentrations given refer to the single 100 µl aliquots. Aliquots of 100 µl of each of the following ice-cold solu-

tions were pipetted in quadruplicate into ice-cold (0°C) polyethylene tubes (Eppendorf Micro test tubes, Eppendorf Gerätebau, Hamburg, F.R.G.).

1. Brush-border membrane fraction resuspended in buffer 2 (0.25 M sucrose/0.01 M triethanolamine-HCl, pH 7.4; membrane protein concentration 992.6 μg/ml).

2. Buffer 2 concentrated twice (0.50 M sucrose/0.02 M triethanolamine-HCl, pH 7.4) with or without inhibitor substances in various concentrations: cytochrome *c* (183, 243, 609, 1218 μM) or 501 μM myoglobin or 501 μM β-lactoglobulin A, respectively.

After pipetting these samples were shaken in a rotary shaker for 3 min (Eppendorf Microsystem) and then the third aliquot was added.

3. ^{125}I-labelled egg-white lysozyme together with unlabelled lysozyme in doubly distilled water. The following lysozyme concentrations (3, 36, 72, 144 μM) were used.

The test tubes were then shaken in a rotary shaker for exactly 3 min and allowed to stand in ice for 2 min, and then centrifuged at $43000 \times g$ for 10 min in the Sorvall centrifuge using a SS-34 rotor with DuPont tubes. The supernatants were carefully removed with a pipette and the entire supernatant (together with the pipette tip) were counted for radioactivity in a Packard Auto-Gamma scintillation spectrometer (model CP 153) using scintillation vials filled with 10 ml of distilled water and shaken for 20 s. The remaining pellet plus the tube was counted in a similar way. The activities of supernatant and pellet added reached a recovery of $96.77 \pm 3.44\%$ ($n = 318$) compared to standards.

In our previous lysozyme binding studies [12] the calculation of the binding data was carried out on the basis of measurements of the dead space. The dead space was determined by using D-[^{14}C]mannose as a reference substance, which is known not to be bound to either brush-border membranes or the plastic tubes. In the previous study [12] no significant adhesion of lysozyme to the polyethylene tubes was found in the presence of the brush-border membranes. To control this possible non-specific adhesion at various lysozyme concentrations and consequently to improve our actual calculation of the binding data, additional experiments were performed. At all lysozyme concentrations used in the presence and absence of inhibitory proteins some samples were equally treated but not centrifuged. In these uncentrifuged samples in the average $6.06 \pm 3.02\%$ ($n = 274$) of the total radioactivity was found within the tube after removing the incubation medium with a pipette. These percentage values amounted up to 10.10% in the absence of inhibitors at the lowest lysozyme concentrations and down to 2.33% in the presence of myoglobin at the lowest lysozyme concentrations. The average test tube counts of uncentrifuged samples at a given lysozyme concentration with or without inhibitor represented the dead space and the non-specific adhesion and were subtracted from the respective pellet (together with the test tube) counts of centrifuged samples in the present calculation.

In control experiments the pH value of the incubation medium was measured and found to be slightly altered in the presence of the highest concentrations of cytochrome *c* and β-lactoglobulin A. These slight changes in the pH values have no influence on lysozyme binding as measured by altering the pH value of the buffer solution in the absence of inhibitor protein.

The efficiency of the centrifugation step in the binding assay was controlled in samples without labelled lysozyme. The supernatant contained less than 1% of the concentrations of both membrane protein and alkaline phosphatase of the uncentrifuged sample.

Test substances

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-Buchler, Braunschweig, F.R.G.) as previously published [22]. 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 0.6 μM. Unlabelled lysozyme was added to the test solutions to achieve the desired lysozyme concentrations. As shown previously [12] a 100-fold change in the specific activity has no influence on lysozyme binding. The enzymatic activity of the labelled lysozyme was always more than 85% of that of unlabelled lysozyme.

The free iodine in ^{125}I-labelled lysozyme solu-

tions was always less than 1% as determined via trichloracetic acid precipitation. As shown previously [12] free iodine did not increase during the binding studies under our experimental conditions.

Cytochrome *c* (research grade, from horse heart) was obtained from Serva, Heidelberg, F.R.G., β-lactoglobulin A (from bovine milk) and myoglobin (type III, from horse heart) from Sigma.

The p*I* values of the purchased batches of proteins were measured as previously described [9]: lysozyme (mol. wt. 14000) p*I* 11.0; cytochrome *c* (mol. wt. 12300) p*I* 10.6; β-lactoglobulin A (mol. wt. 18360) p*I* 5.2; myoglobin (mol. wt. 17800) p*I* 7.3.

Calculations

Mean value ($\bar{x}$), standard deviation (S.D.) and regression analysis by method of least squares were used for statistical analysis of the results. All calculations were performed on a Programmable Electronic Calculator SR 56 (Texas Instruments, Dallas, TX, U.S.A.).

Results

The amount of bound lysozyme increased linearly with increasing membrane protein concentration. At higher membrane concentrations the lysozyme binding approached a constant value (Fig. 1). With increasing free concentration of lysozyme the amount of bound lysozyme/mg membrane protein approached saturation under equilibrium conditions. The Scatchard plot [23] revealed a single binding component type with a dissociation constant of 5.3 μM and of 53.0 nmol binding sites/mg membrane protein (Fig. 2). The cationic low molecular weight protein cytochrome *c* inhibited the ^{125}I-labelled lysozyme binding to the brush-border membranes competitively as in-

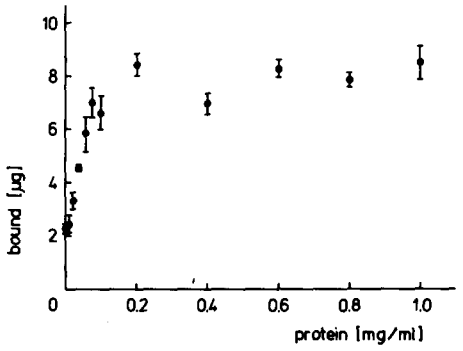

Fig. 1. Binding of ^{125}I-labelled lysozyme at a final lysozyme concentration of 40 μg/ml as a function of membrane protein concentration in the incubation medium. ⧫ represents the mean value ± S.D. of four separate observations.

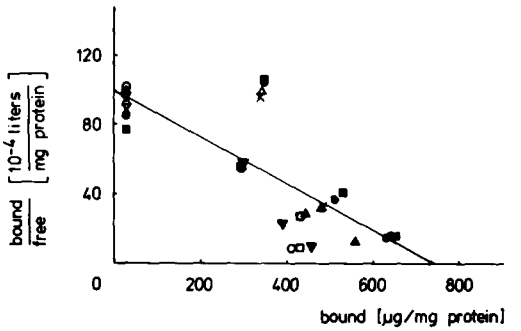

Fig. 2. Binding of ^{125}I-labelled lysozyme to brush-border membranes (Scatchard plot). Different symbols are used for eight separate experiments. Each symbol represents the mean value of four separate observations. The line represents the mean regression curve ($y = -0.1345x + 99.87$; $r^2 = 0.629$).

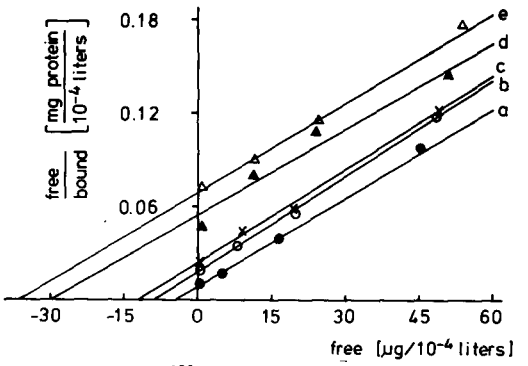

Fig. 3. Binding of ^{125}I-labelled lysozyme to brush-border membranes (Hanes plot) in the absence and presence of the following final concentrations of cytochrome *c*. Each symbol represents the mean of four separate observations. The lines represent the regression curves.

a, ●———●, cytochrome *c* absent
($y = 0.00196x + 0.00810$ $r^2 = 0.9989$)
b, ○———○, 61 μM cytochrome *c*
($y = 0.00204x + 0.01746$ $r^2 = 0.9981$)
c, ×———×, 81 μM cytochrome *c*
($y = 0.00199x + 0.02323$ $r^2 = 0.9906$)
d, ▲———▲, 203 μM cytochrome *c*
($y = 0.00183x + 0.05429$ $r^2 = 0.9566$)
e, △———△, 406 μM cytochrome *c*
($y = 0.00189x + 0.06906$ $r^2 = 0.9995$)

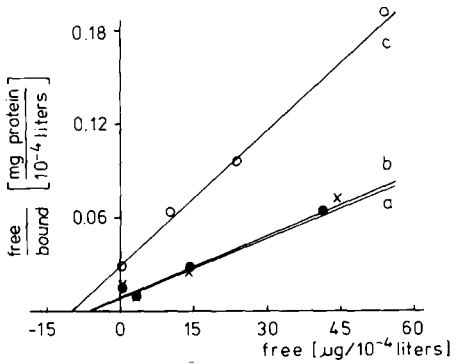

Fig. 4. Binding of ^{125}I-labelled lysozyme to brush-border membranes (Hanes plot) in the absence and presence of myoglobin or β-lactoglobulin A given as final concentrations. Each value represents the mean value of four separate observations. The lines represent the regression curves.

a, ●———●, absence of protein
($y = 0.00135x + 0.00846$ $r^2 = 0.9905$)
b, ×———×, 167 µM myoglobin
($y = 0.00140x + 0.00871$ $r^2 = 0.9757$)
c, ○———○, 167 µM β-lactoglobulin A
($y = 0.00298x + 0.02913$ $r^2 = 0.9979$)

dicated by parallel regression lines in the Hanes plot [24] shown in Fig. 3. The anionic β-lactoglobulin A inhibited lysozyme binding in a noncompetitive manner, while the neutral myoglobin at an equimolar concentration had no effect (Fig. 4).

Discussion

The binding of lysozyme to isolated brush-border membranes was rapid, saturable, reversible, proportional to the membrane protein concentration and characterized as a single-component binding site as also shown in our recent study [12]. The dissociation constant is essentially the same in both binding studies, whereas the number of binding sites/mg membrane protein found in the current work is somewhat higher. This may be due to the higher degree of purification of the present brush-border membrane preparation as indicated by higher enrichment factors for brush-border enzymes and very low specific activities of non-brush-border marker enzymes.

In our previous study [12] the binding of the cationic lysozyme to isolated brush-border membranes was inhibited by cationic amino acids, while neutral amino acids had no effect. The inhibitory effect of cationic lysine was shown to be competitive. In the present study the binding of the cationic lysozyme was inhibited competitively by the cationic cytochrome *c*, anionic β-lactoglobulin A inhibited lysozyme binding in a noncompetitive manner, while neutral myoglobin had no effect. Thus, the effects of cationic and anionic amino acids and small proteins on lysozyme reabsorption [6,9,10,25] can be explained on the basis of their effect on lysozyme binding. These studies suggest a charge-dependent competition between cationic substances at the brush border binding sites as the initial endocytic step, but do not necessarily exclude additional interactions between cationic substances on subsequent steps in the endocytic process. The brush-border binding of the cationic polypeptide aprotinin [27,28] and the cationic aminoglycoside gentamicin [28] was also suggested to be a charge-dependent interaction. The cationic guanidyl derivative was bound to the same extent as the native aprotinin, whereas the neutral tetramaleolyl derivative was not bound at all [27]. In addition to net molecular charge, other factors undoubtedly regulate the extent of macromolecules binding to brush-border membranes, and taken together, these processes may confer specificity and selectivity upon the endocytic uptake process.

Very little is known about the location and distribution of the anionic sites at the cell surface. Endocytic vesicles isolated from rat kidney cortex and probably the area of the brush-border membrane from which they are derived differ markedly from the brush-border microvilli regarding enzymatic content and biochemical composition [29,30]. Endocytic vesicles contain twice as much acidic phospholipids (phosphatidylinositol and phosphatidylserine) but only half as much sphingomyelin as the brush-border microvilli [30]. Thus, the brush-border membrane seems to be not homogeneous in various aspects including the distribution of the negatively charged phospholipids. It was tempting to speculate that the negatively charged headgroups of these phospholipids may act as binding sites for cationic molecules [30].

Since endocytic invaginations start exclusively at the crypt-regions of the brush-border membrane

the question arises how anionic sites at the microvilli are related to the endocytic uptake process. In baby hamster kidney cells ferritin-bound anionic sites are apparently able to redistribute by migration from the surface of microextensions to the surface of the cell body [31]. In contrast, by using intestinal epithelium there was no evidence for movement of ferritin-bound anionic sites along the microvilli [32]. Thus, the intestinal and our previous study showing different composition of the microvilli membrane versus endocytic vesicle membrane including different turnover rates of membrane components [29,30] support the assumption that cationic proteins may move from one anionic binding site to another along the microvilli to the crypt-region.

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