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The binding of beta-2-microglobulin to renal brush-border membrane: affinity measurement, inhibition by serum albumin

Helene Simonnet [a], Catherine Gauthier [a], Claude Vincent [b] and Jean-Pierre Revillard [b]

[a] *Laboratoire de Physiologie, U.F.R. Médicale Alexis Carrel, Lyon and* [b] *INSERM U 80, CNRS UA 1177, UCBL, Hôpital Edouard Herriot, Lyon (France)*

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In the kidney, filtered proteins are rapidly reabsorbed so that the final excretion is less than 0.1% of the filtered amount for low molecular weight proteins such as β_2-microglobulin and a few percent for albumin. In order to investigate the affinity of proteins for luminal membranes, rat renal brush-border membranes were incubated with ^{125}I-labelled human β_2-microglobulin and the initial binding rate determined by the filtration method. Scatchard plot analysis of binding rate revealed two types of binding sites: one with $K_m = 0.25 \cdot 10^{-6}$ M and $V_{max} = 0.1$ nmol/min per mg protein and another with $K_m = 1 \cdot 10^{-5}$ M and $V_{max} = 1.3$ nmol/min per mg protein. The lower affinity type is likely to represent non-specific binding the physiological role of which is to be discussed. The higher affinity sites seem to play the major role in binding rate. β_2-Microglobulin initial binding is reversible, and inhibited by bovine serum albumin. Comparison of the time course of bound β_2-microglobulin removal by unlabelled β_2-microglobulin and by albumin suggests that these two proteins have a different internalization mechanism.

Introduction

Low molecular weight plasma proteins, like hormones, enzymes or immunoglobulin fragments, are able to cross the renal glomerular filter with a sieving coefficient close to 1. Thus they have a short plasma half-life and a rapid turnover [1,2]. Filtered proteins are readily taken up by the tubular cells, and degraded in lysosomes into their amino-acid constituents which return to the plasma compartment, so that the final renal excretion of low molecular weight proteins is less than 0.1% of their filtered amount [3,4]. Conversely serum albumin (M_r 68 000) has a very low sieving coefficient (0.1%), and from 4 to 10% of the filtered fraction is excreted [5]. Histological studies have shown that proteins penetrate the tubular cell via endocytosis vesicles. However, data regarding the affinity of protein binding are needed to explain the overall uptake velocity. From clearance studies, it was hypothesized that bovine serum albumin, and to a lesser extend human serum albumin, were potent competitive inhibitors of the binding of low molecular weight proteins to luminal membranes [3]. Our own studies used similar methods and suggested that competition between rat albumin and human β_2-microglobulin (β_2m)

Correspondence: H. Simonnet, Laboratoire de Physiologie, U.F.R., Médicale Alexis Carrel, rue Guillaume Paradin, 69372 Lyon Cedex 2, France.

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involved at least two levels demonstrable at different time intervals after β_2-microglobulin infusion [6,7]. Recently we described an in vitro assay for binding of labelled β_2-microglobulin to rat renal brush-border membranes [8]. In the present report we define the appropriate analytical method for measuring the affinity of β_2-microglobulin for renal brush-border membrane and the competition between β_2-microglobulin, albumin and other proteins of low molecular weight. Special attention will be paid to kinetic parameters and reversibility of binding.

Methods

Preparation of brush-border membranes

Brush-border membranes were obtained from the renal cortex of male WISTAR rats weighing 300–400 g by a modification of $MgCl_2$ precipitation method of Booth and Kenny [9] as previously described [8]. Aliquots of the obtained suspension were stored in liquid nitrogen until use.

Assessment of brush-border preparation

Protein concentration was determined according to the method of Lowry et al. [10]. The marker enzymes chosen for assessment of the quality of the preparation were the following: aminopeptidase M (EC 3.4.11.2), alkaline phosphatase, acid phosphatase, glucosaminidase (EC 3.5.1.33), β-glucuronidase (EC 3.2.1.31), Na^+/K^+-ATPase (EC 3.6.1.3), succinate–cytochrome-*c* reductase (EC 1.3.99.1) and NADPH–cytochrome-*c* reductase (EC 1.6.2.4). Activities of the enzymes were determined as previously described [8]. Mean enrichment in aminopeptidase was 9.5. The brush-border preparation was essentially free of microsomes and mitochondria. Lysosomial contamination, as assessed by β-glucuronidase activity, was negligible. The main contaminants were acid phosphatase activity (enrichment 1.9) and Na^+/K^+-ATPase (enrichment 1.1).

β_2-Microglobulin labelling

Human β_2-microglobulin ($M_r = 11\,800$) was isolated from urine of several renal transplanted patients according to the procedure of Vincent and Revillard [11] and labelled as described before [8]. Specific activity was 0.75 μCi/μg ($9.9 \cdot 10^5$ cpm/μg). The labelled β_2-microglobulin preparation (30 to 40 μM) was supplemented with 10 μg purified bovine serum albumin per μg β_2-microglobulin. The final preparation was dialysed against 0.15 M NaCl, 0.066 M phosphate buffer (pH 7.4) and stored frozen.

Other proteins

Bovine serum albumin (fraction V, Sigma) was chromatographed on ACA 44 gel (100 × 2.5 cm). 50 mg were included in each run, proteins eluted with 30 mM Tris-HCl buffer (pH 7.8) and 0.1 M NaCl, at 20 ml/h. The albumin fraction was collected then dialysed against final buffer appropriate for further utilization. α_1-Microglobulin (α_1m) (M_r 31 000) was purified as previously described [12]. Insulin (M_r 5700) and myoglobin I (M_r 16 850, from horse skeletal muscle) were obtained from Sigma (Ref. No. I 5500 and M 0630, respectively).

β_2m binding assay

^{125}I-β_2-microglobulin binding to brush-border membranes was determined by the rapid filtration technique as previously described in detail [8]. Briefly 20 μl of membrane suspension (containing about 5 μg protein) in 80 μl buffer (150 mM NaCl, 10 mM Hepes (pH 7.4)) were pre-incubated for 10 min at 37 °C. Binding was initiated by addition of 20 μl incubation medium containing labelled β_2-microglobulin (final content 200 nM) and, when necessary, unlabelled β_2-microglobulin or competitor protein. Final content in albumin and phosphate brought with labelled β_2-microglobulin was 23 μg/ml and 0.3 mM, respectively. After 1 min at 37 °C incubation of β_2-microglobulin was stopped with 2 ml cold stop solution (150 mM choline chloride, 10 mM Hepes (pH 7.4), 20 g/l bovine serum albumin (fraction V)) and the entire tube content was rapidly filtered through a 0.2 μm cellulose acetate filter. Correction for non-specific binding to filter was done by subtracting from each value that of the blank obtained by filtration of a similar incubation mixture, the binding being stopped at time zero prior to addition of labelled β_2-microglobulin. Radioactivity bound to filters was counted in a Packard gamma scintillation spectrometer. Assays were made in triplicate unless otherwise indicated.

Removal experiments

In removal experiments, ^{125}I-β_2-microglobulin was first bound to brush-border membranes following the above-described procedure. After 1 min incubation time, unlabelled β_2-microglobulin or albumin in 200-fold molar excess was added to the medium under a volume of 10 μl and the incubation carried on until the indicated time. The reaction was then stopped as described above.

Mathematical analysis

Eadie-Scatchard plot [13] parameters were determined by a best fit graphical analysis using an iterative computer scanning of the identity:

$$v = v_1 + v_2 = \frac{\frac{V_{max1}[S]}{K_{m1}}}{1+\frac{[S]}{K_{m1}}} + \frac{\frac{V_{max2}[S]}{K_{m2}}}{1+\frac{[S]}{K_{m2}}}$$

where v = total binding rate, v_1 = binding rate to type 1 sites, v_2 = binding rate to type 2 sites, [S] = ligand concentration, V_{max1} = maximal binding rate to type 1 sites, V_{max2} = maximal binding rate to type 2 sites, K_{m1} = Michaelis constant of type 1 sites, K_{m2} = Michaelis constant of type 2 sites.

Results

In order to measure the affinity of β_2-microglobulin for renal brush-border membranes, the relationship between initial binding rate of β_2-microglobulin to membrane and β_2-microglobulin concentration was determined as shown in Fig. 1. v versus s plot (Fig. 1A) shows that binding is saturable and Eadie-Scatchard plot ($v/s = f(v)$, Fig. 1B) displays two groups of points showing that two types of binding sites for β_2-microglobulin may be distinguished on brush-border membranes. The first type is characterized by a high affinity ($K_m = 0.25 \cdot 10^{-6}$ M) and a low capacity (V_{max} = 0.1 nmol/min per mg protein), while the second has low affinity ($K_m = 1 \cdot 10^{-5}$ M) and high capacity (V_{max} = 1.3 nmol/min per mg protein).

β_2-Microglobulin binding to brush-border membranes is partially but quickly reversible as shown in Fig. 2. Following addition of 200-fold

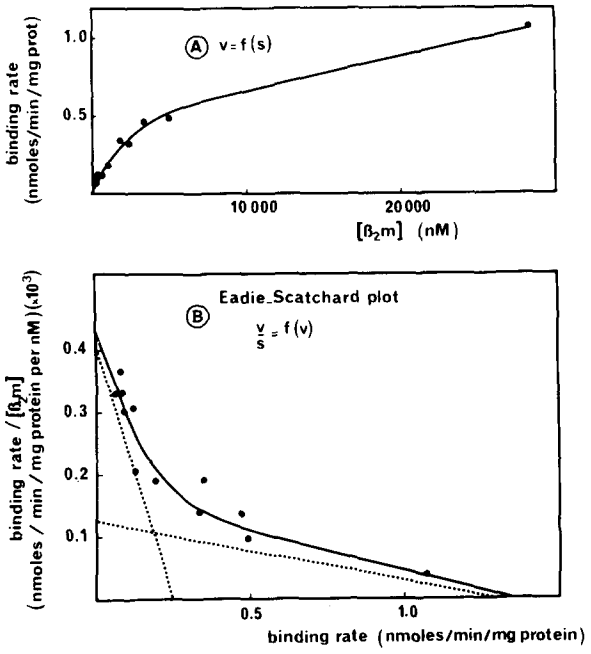

Fig. 1. Kinetics of β_2-microglobulin binding to renal brush-border membranes. The relationship between initial velocity of binding (v, expressed in nmol bound β_2-microglobulin per min per mg protein) and β_2-microglobulin concentration (s, expressed in nM) is plotted as v versus s (A) and as v/s versus v (B). Initial velocity of binding was determined with a 1 min incubation time as indicated in chapter Methods. Final content in β_2-microglobulin was achieved by adding a constant amount of labelled β_2-microglobulin to variable amounts of unlabelled β_2-microglobulin. Specific activity of total β_2-microglobulin (unlabelled and labelled) was calculated for each β_2-microglobulin (β_2m) concentration in order to determine binding values in nmoles. Binding data are representative of two experiments with two preparations.

excess cold β_2-microglobulin to incubation medium after 1 min incubation of membranes with labelled β_2-microglobulin, 50% of bound radioactivity was removed within 30 s. However, despite the presence of an excess of unlabelled β_2-microglobulin, 30 s later labelled β_2-microglobulin progressively bound again to membranes and reached its initial binding level after 30 min. In order to test whether this latter incorporated radioactivity represented bound or transported molecules, in one control experiment (not shown), the incubation mixture was sonicated for 10 s before filtration (Branson cell disruptor B 15, output intensity 5). The overall binding decreased but the general features of the removal curve did not

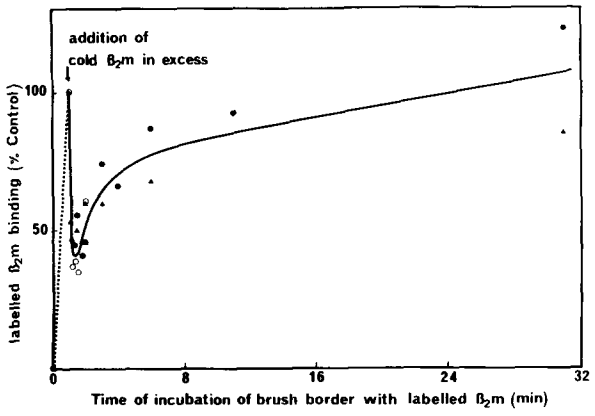

Fig. 2. Removal of bound labelled β_2-microglobulin by unlabelled (cold) β_2-microglobulin (β_2m) in excess of 200 times. 5 μg brush-border membranes were incubated as described in Methods in the presence of 200 nM labelled β_2m under 120 μl. After 1 min, 10 μl unlabelled β_2m in final excess of 200 times versus labelled β_2m were added to the incubation medium and incubation was carried until the time indicated. Labelled β_2m binding in pmol/mg protein was determined. Data from three experiments are plotted versus control binding before addition of unlabelled β_2m.

change. Thus the radioactivity incorporated into brush border was still probably bound to the membrane after 30 min.

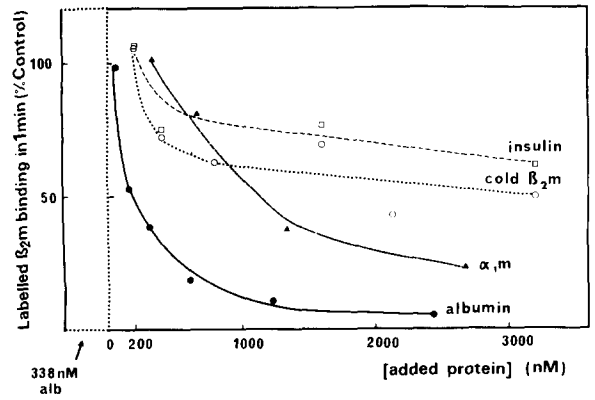

Fig. 3. Inhibition of labelled β_2-microglobulin (β_2m) binding by proteins. 5 μg brush-border membranes were incubated as described in Methods in the presence of 200 nM labelled β_2m and several other proteins in indicated molar concentration; furthermore 338 nM albumin was systematically brought with labelled β_2m preparation. □, Insulin (M_r 5700); ○, unlabelled β_2m (M_r 11800); ▲, α_1-microglobulin (M_r 31000); ●, albumin (M_r 68000). Binding was calculated in pmol/mg brush-border protein (incubation time: 1 min) then expressed in percent of labelled β_2m binding in the absence of added proteins. Data are representative of at least two experiments with two preparations.

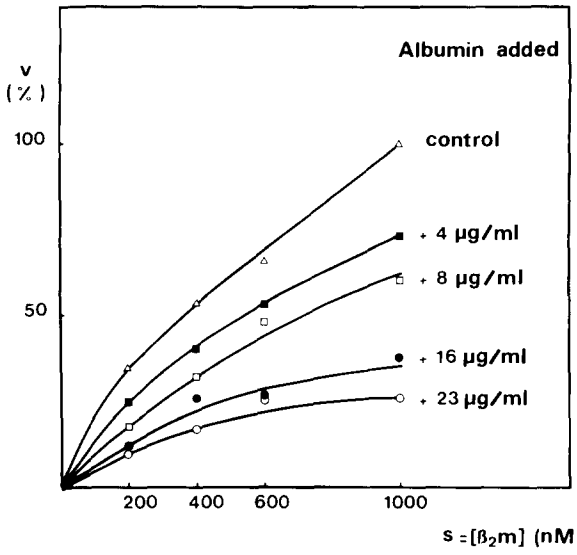

Fig. 4. Effect of bovine serum albumin on β_2-microglobulin (β_2m) binding. Brush-border membrane suspension (50 μg protein/ml) was incubated with β_2m 200 nM, 400 nM, 600 nM or 1000 nM in the presence of varying concentrations of pure bovine serum albumin. Final content in β_2-microglobulin was made up by adding varying amounts of cold β_2-microglobulin to a constant amount of labelled β_2-microglobulin (200 mM) containing 23 μg/ml pure bovine serum albumin (control assay). Final content in albumin was modified by adding the indicated amounts of pure bovine serum albumin. Binding assay was carried out as described in Methods. Each assay was made in quadriplicate. v = binding rate was measured in pmol β_2-microglobulin bound per min per mg membrane protein, then expressed as a percentile of control binding rate with 1000 nM β_2-microglobulin. Data are drawn from five experiments.

Fig. 3 shows that β_2-microglobulin binding was strongly inhibited by bovine serum albumin and to a lesser extent by α_1-microglobulin (M_r 31000), unlabelled β_2-microglobulin (M_r 11800) and insulin (M_r 5700). On the other hand myoglobin (M_r 16800) failed to inhibit β_2-microglobulin binding (not shown on Fig. 3). Bovine serum albumin (M_r 68000) was much more inhibitory than cold β_2-microglobulin itself at equivalent molar concentration. Each assay contained 338 nM of ballast bovine serum albumin brought with the labelled β_2-microglobulin (200 nM). Fig. 3 shows that an inhibition of 50% versus control could be obtained with a total albumin content of about 450 nM which is still close to the normal rat albumin concentration in tubular fluid (15–30

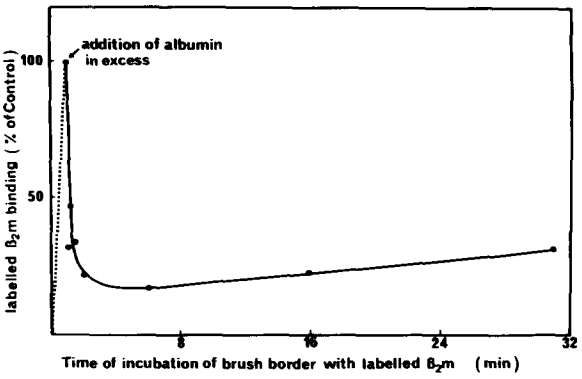

Fig. 5. Removal of bound labelled β_2-microglobulin (β_2m) by bovine serum albumin in weight excess of 200 times. Experimental procedure was the same as in Fig. 2, but unlabelled β_2-microglobulin was replaced by purified bovine serum albumin in weight excess of 200 times versus labelled β_2m. Presented data are representative of three experiments.

μg/ml [15]). The dose effect of albumin was investigated with several concentrations in β_2-microglobulin as shown in Fig. 4. Albumin inhibited β_2-microglobulin binding at any tested concentration. In the region of the K_m for high-affinity sites, addition of 23 μg albumin per ml caused a 70% inhibition. Addition of large excess of albumin to the incubation medium after one minute incubation time of β_2-microglobulin with brush-border membrane resulted in the nearly complete removal of bound β_2-microglobulin (Fig. 5) with a classical exponential type curve.

Discussion

Determining the affinity of β_2-microglobulin for brush-border membrane raised several difficulties preventing the use of the experimental conditions usually employed in classical binding studies. Most of these concerning hormones and antibodies have been carried out in large excess of albumin in order to prevent adsorption to glassware and to reproduce the biological conditions of molecules in plasma. Because kidney epithelial membrane is normally in contact with very small amounts of albumin, and in order to investigate competition among several proteins, albumin concentration had to be minimized in our study. However, to avoid binding to glassware and autoirradiation of labelled β_2-microglobulin, a minimal amount of albumin was required. The final concentration of bovine serum albumin was 23 μg/ml in the standard assay. It is in the range of rat albumin concentration in normal tubular fluid (15–30 μg/ml [5,14]). Measurement of non-specific binding of β_2-microglobulin to membrane proved to be difficult. A mathematically correct determination of non specific binding (which is classically assumed to be non saturable binding) would require to measure the binding of labelled β_2-microglobulin in the presence of an excess of about 10000-times the K_m for β_2-microglobulin, that is 30 g/l. Non-specific binding in this range of concentration is probably also saturated, thus forbidding the classical linear interpolation. For the determination of the affinity constant we therefore chose to measure total binding of β_2-microglobulin, in the expectation of finding at least 2 K_m, allowing a graphical determination of non-specific binding. Actually our results show a biphasic Scatchard plot of binding versus β_2-microglobulin content. The lower affinity type of binding sites (K_m 10^{-5} M) represents probably non-specific binding to the membrane. Another problem was the choice of the incubation time for determining the affinity constant. A general biological system including a binding reaction is well described by the binding of a substrate (S) to an enzyme (E):

$$E + S \overset{1}{\leftrightharpoons} ES \overset{2}{\rightarrow} EP \overset{3}{\rightarrow} E + P$$

where reaction 1 is rapid and reversible and reaction 2 and 3 are slower than reaction 1. Enzymologists generally measure $-d[S]/dt$ or $d[P]/dt$, rate of substrate disappearance or product formation. When reaction 1 is at equilibrium and if (i) measurements are made at initial rate for reaction 2, (ii) complex substrate is only a small fraction of total substrate, (iii) reaction 2 is much slower than reaction 1 so that reversal reaction 2 and 3 are negligible, then $-d[S]/dt = d[P]/dt = k[ES]$ (k is a kinetic constant) and K_m is very close to K_s (or K_d) constant of equilibrium of reaction 1 [13]. Hormonologists measure [E], [S] and [ES]. No reaction 2 or 3 occurs in a simple binding system and K_d (or K_s) the equilibrium constant for dis-

sociation is obtained as soon as reaction 1 is at equilibrium.

In the case of β_2-microglobulin binding to brush-border membranes the time course of binding (see Ref. 8) suggests that binding is at equilibrium after at least 5 min. However, the experiment of removal of bound labelled β_2-microglobulin by unlabelled β_2-microglobulin (Fig. 2) strongly suggests that β_2-microglobulin binding is a complex phenomenon as discussed below. The first event is rapid binding, rapidly reversible, as expected. After one minute affinity is apparently modified since the binding of labelled β_2-microglobulin increases. This experiment could be explained by a binding reaction of the following type:

$$E + S \overset{1}{\leftrightharpoons} ES \overset{2}{\rightarrow} EP$$

where reaction 1 is rapid and reversible and reaction 2 is not reversible. Measured bound radioactivity in this case would reflect the sum of the two complexes ES and EP and equilibrium for reaction 1 would in fact be obtained sooner than 5 min (which is equilibrium of the overall reaction). If reaction 1 is at equilibrium, for initial rate of reaction 2 $d[EP]/dt = k[ES]$. To quantitate the affinity of the simple binding of β_2-microglobulin to membranes we therefore chose to work with a one minute incubation time. This is the longest incubation time with which a reversible binding can be observed (Fig. 2). Doing that, and assuming that β_2-microglobulin binding is a complex phenomenon we worked with an experimental system close to that of enzyme kinetic studies: initial rate of binding for the overall system as measured with a time course experiment, and small fraction of β_2-microglobulin under the form of a complex [8]. Nevertheless since no evidence is available that reaction 1 is at complete equilibrium after one minute, the measured constant K_m is only an apparent K_d.

The value of the lowest K_m we obtained ($0.25 \cdot 10^{-6}$ M) is close to β_2-microglobulin normal tubular fluid content in rat ($0.4 \cdot 10^{-6}$ M) and in human ($0.2 \cdot 10^{-6}$ M). In this range of concentrations, total rate of binding in one minute is mainly due to high-affinity binding sites: we can calculate as detailed in Methods that if $s = 0.25 \cdot 10^{-6}$ M, then v_1 (higher affinity site) = 50 pmol/min per mg and v_2 (lower affinity sites or non-specific) = 31 pmol/min per mg. The rapid binding is quickly reversible showing that the first step of β_2-microglobulin uptake by the tubular cell is a simple binding to high-affinity sites. Lower affinity sites ('non-specific' sites) possibly include several types of sites that cannot be graphically distinguished among which could be brush-border proteinases and some other membrane components to which β_2-microglobulin binds with an even lower affinity. A previous work on lysozyme binding to brush-border showed only one K_d of $0.5 \cdot 10^{-5}$ M [15]. This is possibly explained by a lysozyme specific K_d too close to non-specific K_d to be distinguished, or by different conditions of incubation (0 °C, higher concentration in brush-border membranes) which favor measuring of non-specific binding. The works of Talor et al. [16] and Rabkin et al. [17] on insulin binding to brush borders suggest that, in addition to low-affinity sites, brush border possesses sites with very high affinity (K_d of about 10^{-10} M) and high specificity for insulin. These sites were similar to basolateral sites and the rate of binding was very low. Park and Maack [18] studying albumin reabsorption by isolated perfused proximal convoluted tubules of the rabbit also found two K_m for albumin which were of the same magnitude as ours for β_2-microglobulin. However, the respective contributions of high- and low-affinity sites in in vivo β_2-microglobulin reabsorption rate are still to be clarified. Proteins nonspecifically bound to luminal membrane could be internalized by simple adsorptive endocytosis. However, the works of Bode et al. [19] and Rodman et al. [20] showed that the totality of the brush-border membrane is not internalized by endocytosis. Aminopeptidase, for instance, remains at the surface of the cell, so that a restructuration of the membrane occurs during internalization. A previous study [8] showed that β_2-microglobulin binding rate was in good keeping with reabsorption rate. However, reabsorption rate seemed too high for being accounted for by simple endocytosis, suggesting a clustering process and possibly a major role of high affinity sites in reabsorption rate. Albumin reabsorption rate which is slower than that of low

molecular weight proteins [3,18] could be accounted for by simple endocytosis [18].

Competition among low molecular weight proteins or between albumin and low molecular weight proteins for their renal tubular reabsorption has for long been studied in living animal or in isolated kidneys [21,22,6,3] but remains unclear because of the occurrence of several steps in the course of the reabsorption process [6]. The study of Shottke et al. [15] showed that competition may occur at the membrane binding level between lysozyme and cytochrome *c*, whereas lysozyme binding was non-competitively inhibited by β-lactoglobulin A. Our experiments showed that β_2-microglobulin binding was inhibited by proteins of different molecular weight such as insulin, β_2-microglobulin, α_1-microglobulin and albumin. However, absence of inhibition by myoglobin seems to indicate that several pathways might coexist for protein internalization, as already suggested [6]. Low molecular weight proteins were less inhibitory than albumin, suggesting that molecular weight or the number of possible reactive sites on the molecule may play a role in affinity. Fig. 4 shows that albumin inhibits β_2-microglobulin binding to high-affinity sites: actually 23 μg/ml albumin (338 nM) allowed to obtain a 70% inhibition in the region of K_m for high-affinity sites (250 nM). Higher doses led to an almost complete inhibition (Fig. 3). Since it was calculated above that at this concentration low affinity sites accounted for only 38% of total binding, high-affinity sites proved to be implicated in albumin inhibition. Large scattering of the points in this experimental approach did not allow to determine with accuracy the type of inhibition of β_2-microglobulin binding by albumin. Reversal of binding by albumin (Fig. 5) suggests that these two proteins share a common site of binding. A more conclusive result would be provided by studies on the isolated receptor. Viau et al. [3] also demonstrated an in vivo inhibition of human β_2-microglobulin reabsorption in rat by bovine serum albumin, human serum albumin being much less inhibitory. Though this study did not prove inhibition to be localized at the membrane level [7], it calls attention to possible differences among albumin species with regard to their binding.

The time course of removal of bound β_2-microglobulin by unlabelled β_2-microglobulin and by albumin was investigated. Removal of labelled β_2-microglobulin by unlabelled β_2-microglobulin (Fig. 2) was a biphasic phenomenon with a classical rapid removal (as could be expected after a rapid binding) followed by an increase of binding of labelled β_2-microglobulin in spite of the presence of a 200-times excess of unlabelled β_2-microglobulin. When unlabelled β-microglobulin was replaced by albumin, the time course of removal was a classical exponential type decrease (Fig. 5), differing from the kinetics of removal by unlabelled β_2-microglobulin. These results argue in favor of a two step phenomenon when β_2-microglobulin binds to the membrane: the first is a rapid, reversible binding of β_2-microglobulin on a high-affinity site, which can be inhibited by albumin; the second step would be a slower phenomenon, somewhat enhancing β_2-microglobulin binding and for which no competition occurs between β_2-microglobulin and albumin. To explain the second step phenomenon one may speculate that occupied binding sites undergo a further transformation, possibly clustering, which facilitates the first step of binding or shifts the equilibrium. According to this hypothesis, albumin could possibly bind to several sites and 'gelify' them so that a further mobility would be restrained. In conclusion our results show that (i) the lower reabsorption rate of albumin versus low molecular weight proteins is not due to a lower binding affinity as was suggested from in vivo studies [3], (ii) reabsorption of low molecular weight proteins is mediated by a membrane rapid binding step probably followed by a second membrane step accelerating reabsorption rate, (iii) albumin and β_2-microglobulin are competitors for only the first binding step.

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