The Human $\alpha_2$-Macroglobulin Receptor Contains High Affinity Calcium Binding Sites Important for Receptor Conformation and Ligand Recognition*

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The receptor for $\alpha_2$-macroglobulin-proteinase complexes ($\alpha_2$MR) was purified recently, and its binding of ligand was shown to depend on calcium ions (Moestrup, S. K., and Gliemann, J. (1989) J. Biol. Chem. 264, 15574–15577). This paper shows that the 440-kDa human placental $\alpha_2$MR is a cysteine-rich glycoprotein with high affinity calcium binding sites important for receptor conformation; and the relationship between Ca$^{2+}$ concentration and receptor function is presented. Autoradiography showed $^{46}$Ca$^{2+}$ binding to the 440-kDa $\alpha_2$MR blotted onto nitrocellulose from a sodium dodecyl sulfate-polyacrylamide gel. $\alpha_2$MR immobilized on nitrocellulose in the absence of sodium dodecyl sulfate bound $^{46}$Ca$^{2+}$ in the presence of 5 mM Mg$^{2+}$, and 2–3 $\mu$M unlabeled Ca$^{2+}$ was required to displace half of the bound $^{46}$Ca$^{2+}$. The calcium concentration dependence showed upward concave Scatchard plots, and the number of binding sites was estimated to be approximately eight/$\alpha_2$MR molecule.

Binding of calcium did not change in the pH range 6.5–8.0 but decreased at lower pH values. Addition of Ca$^{2+}$ to the medium was necessary for receptor binding of the $\alpha_2$-macroglobulin-trypsin complex, and half of the maximal binding capacity was obtained with about 16 $\mu$M Ca$^{2+}$ at pH 7.8. The requirement for calcium was increased at lower pH values, and half of the maximal $^{46}$Ca$^{2+}$-trypsin binding was obtained with about 30–40 $\mu$M Ca$^{2+}$ at pH 7.0. Monoclonal antibodies were produced against $\alpha_2$MR, and one of them distinguished between the Ca$^{2+}$-occupied and nonoccupied forms. Like Ca$^{2+}$, Sr$^{2+}$ and Ba$^{2+}$ elicited ligand binding affinity and competed for binding with Ca$^{2+}$ in the order Ca$^{2+}$ > Sr$^{2+}$ > Ba$^{2+}$. In conclusion, calcium ions bind specifically to $\alpha_2$MR with high affinity, and it is likely that several sites on the $\alpha_2$MR molecule have to be occupied to elicit the conformation recognizing the ligand.

The tetrameric glycoprotein in human plasma $\alpha_2$-macroglobulin ($\alpha_2$M) and the homologous pregnancy zone protein form stable complexes with a wide variety of proteinases. Each of the 180-kDa subunits in $\alpha_2$M contains an exposed peptide stretch, the bait region, which is recognized and cleaved by several proteinases. This is followed by the cleavage of an internal thiol ester in each subunit and a conformational change leading to the exposure of a previously concealed receptor recognition site in each subunit. Incubation of native $\alpha_2$M with small nucleophiles such as methylamine also leads to cleavage of the internal thiol esters and exposure of the receptor recognition sites (for review, see Ref. 1).

Receptors for $\alpha_2$M-proteinase complexes (referred to as $\alpha_2$MR receptors, $\alpha_2$MR) have been characterized kinetically in fibroblasts (2–4), macrophages (5–7), hepatocytes (8–10), and membranes derived from placental sincytiotrophoblasts (11). $\alpha_2$MR from these cell types recognizes both $\alpha_2$M- and pregnancy zone protein-proteinase complexes (3, 4, 11). Bound complex is rapidly taken up by receptor-mediated endocytosis in hepatocytes from rodents (12, 13) and humans (10), and uptake into these cells largely accounts for the rapid removal of $\alpha_2$M-proteinase complexes from the circulation, occurring with a half-time of 2–3 min in rodents (12, 13). It is not known whether $\alpha_2$MR of the normal human placenta is able to internalize bound ligand rapidly or whether it serves as a point of attachment of the complexes to the surface of the sincytiotrophoblast (11).

We have recently purified $\alpha_2$MR from detergent-solubilized rat hepatic (14) and human placental (15) membranes as an approximately 440-kDa single-chain protein capable of binding the ligand with an apparent $K_d$ of about 400 PM. Ligand binding occurred only in the presence of Ca$^{2+}$, in agreement with previous results using intact cells (2, 9) and membrane preparations (11, 16). The purpose of the present work was to clarify the Ca$^{2+}$ dependence of ligand binding. We show that $\alpha_2$MR binds Ca$^{2+}$ with high affinity, and we quantify the relationship between bound Ca$^{2+}$ and conformation-dependent ligand binding activity.

EXPERIMENTAL PROCEDURES

Preparation of Human Placental $\alpha_2$MR—Membranes were prepared as described previously from placentas obtained after normal deliveries (11). In brief, pieces (1–2 g) of villous tissue were washed in ice-cold phosphate-buffered saline and homogenized at 0 °C in 250 mM sucrose, 10 mM Hepes, 5 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4. The 3,000 × g supernatant (6 min) was centrifuged at 48,000 × g for 40 min and homogenized in 140 mM NaCl, 0.6 mM CaCl$_2$, 10 mM sodium phosphate, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.8. The membranes were solubilized by resuspension in 6 mg of protein/ml in the above buffer containing 0.5% CHAPS (Aldrich). The suspension was stirred for 10 min at 0 °C followed by centrifugation for 40 min at 48,000 × g. The supernatant was recirculated for 16 h at 0 °C through an affinity column with Sepharose-immobilized methylamine-treated $\alpha_2$M (14, 15) using 8 mg
of ligand per g of dry Sepharose. The column was washed with about 100 bed volumes of buffer containing 1% Triton X-100 or 0.8% octyl β-D-glucoside. The protein (referred to as α2MR preparation) was eluted in buffer containing 0.1% Triton X-100 or 0.8% octyl β-D-glucoside and 2 mM EGTA, pH 6.0, and stored at −20°C in the presence of 35% glycerol. The preparation contained the 440-kDa α2MR and small amounts of 40-kDa and 70–90-kDa proteins, as described previously (14, 15).

**Determination of Amino Acid Composition**—Approximately 1 ml of α2MR dissolved in octyl β-D-glucoside-containing buffer (A_20_0 = 0.150) was lyophilized, redissolved in 0.5 ml of 50% formic acid, and gel filtered on a Superose 12 column equilibrated and eluted with 50% formic acid (see Fig. 1). The fractions containing the 440-kDa receptor were combined, and 50-μl aliquots were dried and hydrolyzed with 6 M HCl at 110°C for 20 or 72 h. Some aliquots were also oxidized with performic acid (17) prior to the 20-h hydrolysis, or they were hydrolyzed in the presence of 3,5-dithiodiisopropionic acid (18). For determination of tryptophan, samples were hydrolyzed with 1 M NaOH at 110°C for 20 h. After acidification with acetic acid, the hydrolysates were analyzed using a Hewlett-Packard Amino Quant instrument operated according to the manufacturer's instructions and on an instrument built and operated as described by Barkholt and Jensen (18). Bovine prothrombin fragment 1 containing 4 phenylalanine and 3 tryptophan residues (19) was used to determine the recovery of tryptophan. Details of the methodology are provided in the legend of Table I. Calculation of E_280_1cm from the content of tryptophan, tyrosine, and cysteine was performed according to Gill and von Hippel (20). For calculation of the carbohydrate content, we assumed that the fraction of total sugar provided by aminosugar (glucosamine) was 0.1%.

**Preparation of Monoclonal Anti-α2MR Antibodies**—Male BALB/c mice were immunized intraperitoneally four times at 14-day intervals with about 10 μg of α2MR preparation in 100 μl of affinity column eluent. Three days after the last booster injection, spleen cells were fused with 5 x 10^5 mouse myeloma (NS-1) cells and seeded according to standard procedures (22). Screening for positive wells employed a solid-phase enzyme-linked immunosorbent assay using microtiter plates with about 50 ng of α2MR per well and peroxidase-conjugated rabbit anti-mouse IgG (Dacopatts, Copenhagen, Denmark). Cells were cloned twice from positive wells by limiting dilution, and the class and subclass specificities of the monoclonal antibodies were determined using a commercial mouse typing kit (Bio-Rad 172-2055).

Four monoclonal antibodies (designated A2MR-1 through 4) of the IgG1 class were characterized, and none of them inhibited binding of α2M-trypsin. A description of their binding to different epitopes will appear elsewhere (28).

**Blotting Procedures**—SDS-PAGE was performed according to Sambrook (23) as described previously (14). Transfer of proteins onto nitrocellulose was performed using a semidyed electrotblotter (JKA-Biotec, Copenhagen, Denmark) according to the suggestions of the manufacturer. Ca^2+ binding was detected by the method of Marayama et al. (24). In brief, the nitrocellulose sheets were incubated for 15 min at 4°C with 10 mM Tris, 5 mM MgCl_2, pH 7.4. The sheet was washed three times in distilled water followed by autoradiography for 24–72 h using Hyperfilm (Amersham Corp.). Immunoblot analyses were performed using the peroxidase-conjugated rabbit anti-mouse IgG as the secondary antibody.

**Ca^2+ and ^125I-α2M-trypsin Binding Assays**—For Ca^2+ binding, 2 μl of α2MR preparation was applied to nitrocellulose (Sartorius SM 12752) discs with a diameter of 4 mm. The discs were dried for 30 min at 4°C, and 2 μl was applied to the other side of the disc now containing about 2 pmol of α2MR. The discs were washed three times in 0.01 M KCl, 10 mM Tris, 10 mM glycine, 5 mM MgCl_2, pH 7.8 (if not stated otherwise) and incubated at 4°C in 200 μl of the same buffer with 50 nCi of ^45Ca^2+. The incubations were stopped by rapid washing in 2 × 5 ml of ice-cold buffer, and the discs were assayed for radioactivity in a liquid scintillation counter. Blank values determined with discs not containing α2MR showed association of 0.2% of the Ca^2+ in the 200 μl of incubation buffer. This value was not different from that obtained in the presence of a saturating concentration of α2M-trypsin, but was subtracted from all other values. Omission of MgCl_2 from the buffer increased the blank value to a magnitude prohibiting the measurement of ^45Ca^2+ binding to α2MR. Analyses using atomic spectrophotometry showed that the deionized glass-distilled water used in these experiments contained less than 0.5 μM Ca^2+.

Binding of ^125I-α2M-trypsin was performed using the same assay with the following modifications. About 0.2 pmol of α2MR was applied to the discs followed by soaking in buffer containing 1% Tween 20 to prevent nonspecific absorption of protein. The incubations were performed for 16 h, a time sufficient to achieve a steady state of binding (data not shown). Blank values (1.0–1.5% of the added tracer) were not different from the values obtained in the presence of a saturating concentration of unlabeled α2M-trypsin (140 nM) and were subtracted from all other values.

The following results were obtained in control experiments. ^125I-Labeled α2MR applied to the nitrocellulose discs did not dissociate during the incubation and washing procedures. Soaking of the discs in Tween 20, necessary for obtaining reliable ^125I-α2M-trypsin binding, did not perturb Ca^2+ binding. Previously, α2MR binding activity was measured in 140 mM NaCl, 10 mM sodium phosphate, 0.6 mM Ca^2+, 4% bovine serum albumin, pH 8.0 (14, 15). Saturable binding of ^125I-α2M-trypsin in that buffer (with filters soaked in albumin before the addition of tracer) was not different from the results obtained when using the present buffer. However, Tween 20 was preferred since it gave lower blank values than albumin. No difference in binding activity was observed between α2MR eluted in buffer containing Triton X-100 or octyl β-D-glucoside. Storage at −20°C in the presence of 35% glycerol was important for maintenance of the binding activity, and under these conditions α2MR was stable for at least 6 weeks.

**RESULTS**

**Amino Acid Composition**—Fig. 1 shows the elution profile of the lyophilized α2MR preparation in 50% formic acid. SDS-PAGE shows that the large peak (lane 1) contains only the 440-kDa α2MR whereas the shoulder (lane 2) contains in addition the minor proteins present in the α2MR preparation (15). In several experiments the content of minor proteins was estimated from the elution profile and from gel scannings to be 10–15%.

Table 1 shows the amino acid composition of the 440-kDa α2MR. The content of half-cystine is remarkably high. The only amino sugar was glucosamine, accounting for about 4.5% of the combined molar yield of amino acids. The carbohydrate size was 0.5 ml. SDS-PAGE (5–20% polyacrylamide) shows that the half peak (lane 1) contains only the 440-kDa α2MR whereas the shoulder (lane 2) contains the proteins of lower molecular weight (lane 2, fractions 20 and 21). Molecular size markers were α2M-methylamine (360 kDa), rat α1-inhibitor 3 (200 kDa), and bovine serum albumin (67 kDa).
TABLE I
Amino acid composition and glucosamine content of α2MR

<table>
<thead>
<tr>
<th>Residue</th>
<th>mol %</th>
<th>Residues/molecule</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp + Asn</td>
<td>13.58</td>
<td>448</td>
</tr>
<tr>
<td>Thr</td>
<td>5.68</td>
<td>187</td>
</tr>
<tr>
<td>Ser</td>
<td>7.16</td>
<td>236</td>
</tr>
<tr>
<td>Glu + Gln</td>
<td>9.28</td>
<td>306</td>
</tr>
<tr>
<td>Pro</td>
<td>4.93</td>
<td>163</td>
</tr>
<tr>
<td>Gly</td>
<td>8.53</td>
<td>281</td>
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<tr>
<td>Ala</td>
<td>5.61</td>
<td>185</td>
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<td>Cys</td>
<td>6.36</td>
<td>210</td>
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<tr>
<td>Val</td>
<td>5.88</td>
<td>194</td>
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<tr>
<td>Met</td>
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<td>50</td>
</tr>
<tr>
<td>Ile</td>
<td>4.24</td>
<td>143</td>
</tr>
<tr>
<td>Leu</td>
<td>6.77</td>
<td>223</td>
</tr>
<tr>
<td>Tyr</td>
<td>2.74</td>
<td>90</td>
</tr>
<tr>
<td>Phe</td>
<td>3.11</td>
<td>103</td>
</tr>
<tr>
<td>His</td>
<td>2.66</td>
<td>88</td>
</tr>
<tr>
<td>Lys</td>
<td>4.11</td>
<td>135</td>
</tr>
<tr>
<td>Arg</td>
<td>5.80</td>
<td>191</td>
</tr>
<tr>
<td>Trp</td>
<td>1.93</td>
<td>64</td>
</tr>
<tr>
<td><strong>Sum</strong></td>
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<td>3297</td>
</tr>
<tr>
<td>GlcNH₂⁺</td>
<td></td>
<td>148</td>
</tr>
</tbody>
</table>

a Corrected for an estimated hydrolysis loss of 5%.
b Corrected for an estimated hydrolysis loss of 10%.
c Determined as cystic acid or as a 3,3'-dithiodipropionic acid adduct (Ref. 18).
d Results of hydrolysis for 72 h.
e Results of hydrolysis with 1 M NaOH (110 °C, 20 h).
f Corrected for an estimated hydrolysis loss of 50%.

groups constituted about 17% of the mass of the receptor, leaving about 365 kDa for the peptide moiety (approximately 3300 residues). From the data shown in Table I, E₉₀,₁ cm was determined as 12.5 when using 50% formic acid as solvent (see the legend to Table I). At neutral pH and under non-denaturating conditions E₉₀,₁ cm is probably 5-10% higher, i.e. about 13.5. E₉₀,₁ cm was calculated as 11.5 on the basis of the content of tryptophan, tyrosine, and cysteine (20). We decided to use the mean value of 12.5 since both methods for the determination of E₉₀,₁ cm are subject to errors.

Binding of Ca²⁺—Fig. 2, panel A, shows a nitrocellulose blot of α₂M-methylamine for control (lane 1) and the α₂MR preparation (lane 2). The proteins were blotted from a continuous 33.5% polyacrylamide gel, and the minor proteins in the α₂MR preparation therefore migrated with the front. Panel B shows the results of incubation of the nitrocellulose sheet with ⁴⁵Ca²⁺ followed by washing and autoradiography. The blotted 440-kDa α₂MR bound ⁴⁵Ca²⁺ whereas the minor proteins did not (lane 2), and no binding to α₂M-methylamine was observed (lane 1).

The following experiments were designed to quantify Ca²⁺ binding and the relationship between Ca²⁺ occupancy and α₂M-trypsin binding. For this purpose it was important to use non-denaturing conditions (15) and to employ the same type of assay system for the two measurements. We therefore used the assay with nitrocellulose-immobilized α₂MR preparation and disregarded the presence of the minor proteins since the

440-kDa α₂MR accounts for nearly all Ca²⁺ (Fig. 2) and α₂M-trypsin (15) binding as well as 85–90% of the absorbance at 280 nm (Fig. 1).

Fig. 3 shows that a plateau of binding of 0.3 μM ⁴⁵Ca²⁺ to nitrocellulose-immobilized α₂MR is achieved by about 15 min. The inset shows that dissociation of ⁴⁵Ca²⁺ from the filter occurs quite slowly, leaving enough time for the washing inherent in the assay procedure. However, the rate of dissociation is markedly increased in the presence of EGTA (C).

Effect of Ca²⁺ and pH on α₂M-trypsin Binding—Fig. 5 shows the dependence of ¹²⁵I-α₂M-trypsin binding on the concentration of Ca²⁺ at pH 7.8. No binding was observed when Ca²⁺ was not added to the buffer, and 50–100 μM Ca²⁺ was required
Fig. 4. Concentration dependence of Ca\(^{2+}\) binding. Immobilized \(\alpha_2\)MR (2.0 pmol/disc in 200 \(\mu\)l) was incubated with \(^{45}\)Ca\(^{2+}\) at the indicated Ca\(^{2+}\) concentrations for 15 min at 4 °C. The inset shows the data plotted according to Scatchard. The curve is computed according to the assumption that Ca\(^{2+}\) binds to two independent sites with different affinities. The constants calculated according to this model are: \(K_{d1} \approx 0.62 \mu\text{M}; K_{d2} \approx 12.71 \mu\text{M}; R_{d1} \approx 23.6 \text{nm}; R_{d2} \approx 57.2 \text{nm}, \) where the \(K_d\) values are the apparent affinity constants, and the \(R_d\) values are the Ca\(^{2+}\) site concentrations. Thus, the site concentration is 81 nm with 10 nm \(\alpha_2\)MR, and the receptor contains about eight sites/molecule.

Fig. 5. Effect of Ca\(^{2+}\) on \(\alpha_2\)MR ligand recognition. Immobilized \(\alpha_2\)MR (0.2 pmol/disc) was incubated (200 \(\mu\)l) for 16 h at 4 °C in buffer containing 20 \(\mu\)M \[^{125}\]I-\(\alpha_2\)M-trypsin (\(7^\prime\)) and Ca\(^{2+}\) as indicated. The points are the mean values of four replicates ± 1 S.D.

Fig. 6. Effect of pH on binding of \(\alpha_2\)M-trypsin (\(7^\prime\)) and Ca\(^{2+}\). Immobilized \(\alpha_2\)MR was incubated at the indicated pH values with 20 \(\mu\)M \[^{125}\]I-\(\alpha_2\)M-trypsin for 16 h either in the presence of 1 mM (O) or 32 \(\mu\)M (O) Ca\(^{2+}\) or with 0.25 \(\mu\text{Ci/ml }^{45}\text{Ca}^{2+}\) (A). The results are expressed as percent of the maximal \[^{125}\]I-\(\alpha_2\)M-trypsin binding (pH 7.8) obtained at the given Ca\(^{2+}\) concentration or as percent of the maximal \(^{45}\text{Ca}^{2+}\) binding.

for maximal binding activity. About 16 \(\mu\)M Ca\(^{2+}\) was required for half-maximal activity (17.1 ± 3.9 \(\mu\)M (1 S.D.) in five experiments).

Fig. 6 demonstrates the interrelationship between Ca\(^{2+}\) and proton concentrations. At 1 mM Ca\(^{2+}\), binding of \(^{125}\)I-\(\alpha_2\)M-trypsin increased with increasing pH, and half of the maximal binding was obtained at pH about 7.0. Binding of \(\alpha_2\)M-trypsin was more pH sensitive at submaximal Ca\(^{2+}\) concentrations, and at 32 \(\mu\)M Ca\(^{2+}\), pH 7.4, was required to obtain half the binding observed at pH 7.8. On the other hand, binding of \(^{45}\text{Ca}^{2+}\) remained nearly unchanged in the pH range 8.2-6.5 and decreased at more acidic pH. It is concluded that both Ca\(^{2+}\) binding and deprotonation are required for the binding of \(\alpha_2\)M-trypsin and that the requirement for deprotonation is increased at suboptimal Ca\(^{2+}\) concentrations.

Ca\(^{2+}\)-dependent Reactivity with a Monoclonal Antibody—Three of the antibodies did not distinguish between the Ca\(^{2+}\)-occupied and nonoccupied forms, and results with one of them are demonstrated in Fig. 7A together with an immunoblot showing reaction with the 440-kDa \(\alpha_2\)MR. Fig. 7B shows that one of the antibodies distinguished between the two forms. Separate experiments (not demonstrated) showed identical gel filtration profiles (Sephacryl S-400, pH 7.8) of the \(\alpha_2\)MR preparation in the absence and presence of Ca\(^{2+}\), indicating lack of Ca\(^{2+}\)-induced oligomerization of the receptor. Thus, Ca\(^{2+}\) induces a conformational change in the \(\alpha_2\)MR monomer.

Specificity of the Ca\(^{2+}\) Binding Sites—Several divalent metal ions and La\(^{3+}\) were tested for the ability to elicit \(\alpha_2\)MR ligand binding activity. Fig. 8 shows that in addition to Ca\(^{2+}\), Sr\(^{2+}\) and Ba\(^{2+}\) elicited \(\alpha_2\)M-trypsin binding activity in the order Ca\(^{2+}\) > Sr\(^{2+}\) > Ba\(^{2+}\) whereas the other divalent ions shown in Fig. 8 and La\(^{3+}\) did not. Fig. 9 shows that Sr\(^{2+}\) and Ba\(^{2+}\) competed for binding of \(^{45}\text{Ca}^{2+}\) following the same order. In addition, La\(^{3+}\) was an effective competitor of Ca\(^{2+}\) binding even though it did not elicit \(\alpha_2\)M-trypsin binding activity. Thus, the two ions most closely related to Ca\(^{2+}\) both bind to...
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to (Y~MR although the question obviously cannot be answered
before information on the amino acid sequence is available.

binding to these as well as most other proteins with high
binding activity. Immobilized α₂MR was incubated with ²¹²⁵I-α₂M-trypsin (T) as explained in the legend to Fig. 5 and ions as indicated. None of the ions changed the association of ²¹²⁵I-α₂M-trypsin to the nitrocellulose in the absence of receptor, and data were corrected accordingly.

The results show that efficient binding of α₂M-trypsin
and induce the conformational change necessary for
α₂M-trypsin binding.

The results show that α₂MR is a cysteine-rich glycoprotein
containing multiple binding sites for Ca²⁺. Moreover, occupancy
with Ca²⁺ or related ions induces a conformational change necessary for binding of α₂M-proteinase complex.

The upward concave Scatchard plot shows that the Ca²⁺
binding is complex. The total number of sites may be under-
estimated from such curved plots, and eight Ca²⁺ binding
sites/α₂MR molecule should therefore be taken as a minimum.

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The overall affinity (Kd = 2 μM) is similar to that of sites
in classical Ca²⁺ binding proteins such as calmodulin and
tropomyosin C. Calmodulin binds four Ca²⁺, probably with
pairwise different affinities and mutual interactions (25, 26).
Sr²⁺ interacts with the same sites, although with lower affinity,
and can elicit activation of calmodulin-activated enzymes
(27). Tropomyosin C from skeletal muscle also binds four
Ca²⁺, and two of the sites (Kd = 3 μM) are thought to be
regulatory (28, 29). Recently, a single Ca²⁺ binding site (Kd =
2 μM) in the galactose chemoreceptor protein from
Koscherichia coli was described in detail (30). The structural basis for Ca²⁺
binding to these as well as most other proteins with high
affinity sites is the EF-hand motif (31). This may also apply to α₂MR although the question obviously cannot be answered
before information on the amino acid sequence is available.

The results show that efficient binding of α₂M-trypsin
occurs only when a large fraction of the Ca²⁺ sites is occupied.

Moreover, increasing protonation increases the requirement
for Ca²⁺. What does this mean in physiological terms? As a
minimum, Ca²⁺ is necessary for the proper ligand-binding
conformation of α₂MR. If the Ca²⁺ sites are on the extracel-
ular side of the protein, the Ca²⁺ concentrations should
always be sufficient for optimal ligand binding, even if only
the sites with the lowest apparent affinity are important for
the receptor conformation. However, a decrease in Ca²⁺,
together with decrease of pH, would cause a rapid and efficient
removal of the protein ligand from α₂MR. This probably
happens in the endocytotic vesicle after internalization of the
α₂MR-α₂M-proteinase complex. It seems unlikely that the
sites are facing the cytosolic side since at least 16 μM Ca²⁺
is required for half-maximal expression of ligand binding activ-
ity. However, sites in or near the membrane-spanning region
might be exposed to Ca²⁺ concentrations in the range of 5–50
μM and thus be regulatory.

Electrophysiological studies have shown that α₂M added
extracellularly can induce spikes of hyperpolarization elicited
by opening of Ca²⁺-dependent potassium channels (32). It is
tempting to speculate that this phenomenon is somehow
related to the Ca²⁺ binding of α₂MR and internalization of
the complex. In any case, the role of the Ca²⁺ binding sites,
from apart keeping the protein in the proper conformation,
is unclear, and information on sequence and structure is essen-
tial for clarification.

Little is known about Ca²⁺ binding to other mammalian
cell membrane receptor proteins. The hepatic asialo-glycopro-	ein receptor is reported to bind Ca²⁺ only when occupied with
the protein ligand (33) whereas others have reported Ca²⁺
binding (Kd = 350 μM) to the unoccupied receptor (34). A
related chicken hepatic lectin requires 1–2 mM Ca²⁺ for half-
maximal ligand binding activity at pH 7.8 (35). The low
density lipoprotein receptor requires Ca²⁺ for binding of the
protein ligand (36), but no direct information on Ca²⁺ binding
seems available. The recently cloned low density lipoprotein
receptor-related protein, with possible growth-modulating ef-
fects, binds ⁴⁵Ca²⁺ when blotted onto nitrocellulose (37), but
no information is available on the affinity or location of the
sites.

Thus, as compared with other mammalian cell membrane
receptors, α₂MR has high affinity Ca²⁺ binding sites, and Ca²⁺
occupancy is as a minimum essential for the ligand binding
conformation of the receptor protein. These properties may
provide an interesting model system for studying the effects
of Ca²⁺ binding on protein conformation.

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analysis of nonoxidized hydrolysates.

REFERENCES
J. Biol. Chem. 254, 5153–5160
3. Gliemann, J., Moestrup, S. K., Jensen, P. H., Sottrup-Jensen, L.,
Biochim. Biophys. Acta 883, 400–406
J. Biol. Chem. 261, 16622–16625
7328
6. Petersen, C. M., Rijvers, E., Hansen, P. W., and Gliemann, J.
7. Moestrup, S. K., Christensen, E. I., Sottrup-Jensen, L.,