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# Chaperone Properties of Calreticulin

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Calreticulin is a highly conserved protein with a relative molecular weight of 46 000, and is mainly located in the endoplasmic reticulum. Calreticulin was first characterized as a calcium-binding protein in the endoplasmic reticulum, but since then other functions of calreticulin have been characterized, including chaperone and lectin properties, and regulation of integrin and nuclear hormone receptor activity. We have investigated the interactions between purified human placental calreticulin and native and denatured proteins. Our results show that calreticulin binds to denatured proteins in a time- and pH-dependent manner, which at physiological pH is dependent on divalent cations. The binding was dependent on the state of the denatured protein, and was highly sensitive to the ionic composition of the environment, being strongly inhibited by phosphate-containing compounds. Calreticulin did not seem to distinguish between denatured glycosylated and non-glycosylated proteins, and was found to bind to native basic proteins, presumably by sheer electrostatic forces.

Calreticulin is a highly conserved ubiquitous eukaryotic protein with a calculated relative molecular weight of 46 000.[1,2] Calreticulin contains three domains called N-, P-, and C-domains, and it is mainly located in the endoplasmic reticulum (ER), although it has also been reported to be present in the nuclear envelope and the nucleus, in the cytoplasm, and at the cell membrane.[3–8]

The functions of calreticulin have not been fully elucidated. Calreticulin was first characterized as a calcium-binding protein, with the P-domain containing a low capacity, high affinity $Ca^{2+}$-binding site (1 mol $Ca^{2+}$/mol protein), and the *C*-terminal domain containing a high capacity, low affinity $Ca^{2+}$-binding site (up to 25 mol of $Ca^{2+}$/mol protein), a property which may be important in the regulation of the calcium concentration in the ER.[2,9–11] In addition to binding of $Ca^{2+}$, calreticulin also binds $Zn^{2+}$ with a relatively low affinity.[12]

Chaperone activity is believed to be a major function of calreticulin, in addition to a possible role in the regulation of integrin and nuclear hormone receptor activity.[6,13–16] Calreticulin has been found to bind to partially trimmed, monoglucosylated glycans, and it has been shown that calreticulin's binding involves a direct oligosaccharide–protein interaction specific for monoglucosylated oligosaccharides.[17–24] Calreticulin has also been shown to bind rabbit lung flavin-containing monooxygenase and to bind fully glycosylated myeloperoxidase.[25,26] Moreover, calreticulin seems to interact with other enzymes of the ER including protein disulfide isomerase, the thiol-dependent reductase ERp57, and endomannosidase.[17,27,28] However it is uncertain whether the chaperone activity of calreticulin is related only to its lectin properties or whether the molecule can interact with substrates both as a lectin and as a chaperone with preference for certain amino acid sequences.

A major problem in studies on the properties and functions of calreticulin is the acidic nature of the protein (pI = 4.65), and its highly charged *C*-terminus which is involved in the high capacity, low affinity divalent cation-binding property. Therefore, some of the functions and interactions described for calreticulin may be due to physico-chemical interactions. In an attempt to define in more detail the properties of calreticulin, we have investigated the interactions of calreticulin with native and denatured proteins under different conditions. We report that calreticulin binds to denatured proteins in a time- and pH dependent manner, which, at physiological pH, is dependent on divalent cations, and is very sensitive to the ionic composition of the environment. Moreover, the binding to denatured proteins is highly dependent on the state of the denatured protein.

## Materials and methods

*Chemicals.* Bovine serum albumin (BSA), ovalbumin (OVA), urea, dithiothreitol (DTT), trishydroxymethylaminomethane, mannose, mannose 1-phosphate, mannose 6-phosphate, glycerol, nitro blue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate, *p*-nitrophenyl

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phosphate substrate tablets, *N*-hydroxysuccinimidobiotin, iodacetamide, glycerol, Triton X-100, and Coomassie Brilliant Blue R-250 were from Sigma (St. Louis, USA). 5,5-Diethylbarbituric acid, and *N,N*-dimethylformamide were from Merck (Darmstadt, Germany). Triton X-114, and Tween 20 were from Merck-Schuchardt (Schuchardt, Germany). Alkaline phosphatase-conjugated streptavidin, alkaline phosphatase-conjugated swine immunoglobulins against rabbit immunoglobulins, and horseradish-peroxidase-conjuated streptavidin were from DAKO (Copenhagen, Denmark). Enhanced chemiluminescence (ECL) reagents were from Amersham (Buckinghamshire, UK). SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) molecular weight standard proteins were from Bio-Rad (Hercules, CA, USA). 4–12% Precast polyacrylamide gels, and tris-glycine running buffer were from Novex (San Diego, CA, USA). Human placentas were kindly provided by the maternity ward, Rigshospitalet (Copenhagen, Denmark). Human sera, phosphate-buffered saline pH 7.4 (82 mM NaCl, 43 mM $Na_2HPO_4$, 10 mM $KH_2PO_4$), sample buffer (0.28 M SDS, 260 mM DTT, 35% glycerol, 0.25 M tris-buffer pH 6.8, and 10% pyronin G), alkaline phosphatase washing buffer (154 mM NaCl, 10 mM Tris, 0.5% Tween 20, pH 7.5), and alkaline phosphatase substrate buffer (1 M diethanolamine, 0.5 mM $MgCl_2$, pH 9.8) were from Statens Serum Institut (Copenhagen, Denmark). Nitrocellulose membranes (0.45 μm) were from Schleicher & Schuell (Dassel, Germany). Two-dimensional SDS-PAGE gels, Mono Q columns, and Con A Sepharose (concanavalin A) were from Pharmacia (Uppsala, Sweden). Immuno Plates (maxisorp) were from NUNC (Roskilde, Denmark).

*Purification of human placental calreticulin.* A placenta was homogenised at 4 °C with 0.5 l 0.1 M sodium phosphate, pH 7.2 and then centrifuged for 20 min at 10 000 rpm in a Sorvall centrifuge (GSA rotor). The pellet was homogenised with 0.5 l 0.1 M PB, pH 7.2, and 1% Triton X-114, and centrifuged for 20 min at 10 000 rpm. An equal volume of 0.1 M sodium phosphate containing 1% Triton X-114 was added to the supernatant and phase separation was performed overnight at 37 °C. Ammonium sulfate was added to the water phase until a final concentration of 337 g $l^{-1}$ was attained, and the solution was stirred at 4 °C overnight. The solution was centrifuged for 1 h at 10 000 rpm, and a second round of ammonium sulfate precipitation was performed with the supernatant; 150 g $l^{-1}$ ammonium sulfate was added and pH adjusted by addition of $H_3PO_4$ to pH 4.7, and the mixture was stirred overnight at 4 °C. After centrifugation at 10 000 rpm for 1 h, the pellet was dissolved in 100 ml 5 mM sodium phosphate, pH 7.2 and dialysed three times against 5 mM sodium phosphate, pH 7.2. 1 ml aliquots of the dialysed mixture were chromatographed on an anion exchange column (Mono Q) connected to an FPLC system (fast performance liquid chromatography), using a linear gradient over 60 min from 0 to 1 M NaCl (flow rate: 1 ml $min^{-1}$). The fraction containing calreticulin was identified by analysing the fractions on SDS-PAGE gels, and by ELISA and immunoblotting with rabbit antisera against calreticulin.

*Biotinylation of calreticulin.* The pH of 1.5 ml purified calreticulin was adjusted to 9 with 1 M NaOH, and *N*-hydroxysuccinimidobiotin dissolved in *N,N*-dimethylformamide (60 μl, 10 mg $ml^{-1}$) was added. The solution was mixed end-over-end for 2 h at room temperature. The mixture was dialysed three times against phosphate-buffered saline (PBS), and an equal volume of glycerol was added. The biotinylated calreticulin was stored at −20 °C until required.

*Enzyme-linked immunosorbent assay (ELISA).* The antigens (1 μg $ml^{-1}$) were coated onto the surface of the wells of microtitre plates, using 0.05 M sodium carbonate as coating buffer. Coatings were carried out overnight at 4 °C using 100 μl per well. After coating the plates were washed three times for 1 min with alkaline phosphatase washing buffer. Subsequent incubations with biotinylated calreticulin diluted in incubation buffer (50 mM Tris, 0.5% Tween 20, pH 7, with or without the addition of 10 mM $MgCl_2$ or $CaCl_2$) were carried out for 2 h at room temperature on a shaking table, and were followed by three washes in alkaline phosphatase washing buffer and 1 mM $MgCl_2$. Finally, the plates were incubated 1 h at room temperature with alkaline phosphatase-conjugated streptavidin diluted 1 : 1000 in 0.05 M Tris, 10 mM $MgCl_2$ and 0.5% Tween 20, pH 7.5. After three washes in alkaline phosphatase washing buffer containing 1 mM $MgCl_2$, bound calreticulin was quantitated using *p*-nitrophenyl phosphate in alkaline phosphatase substrate buffer (1 mg $ml^{-1}$). The absorbance was read at 405 nm with background subtraction at 690 nm.

*Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).* SDS-PAGE was performed as described by Laemmli.[29] Samples were mixed with an equal amount of sample buffer and heated at 100 °C for 1 min, and 20 μl were added to the wells of a 4–12% precast Novex gel. The gel was run at 100 V for approximately 90 min. The gel was stained in 0.1% Coomassie Brilliant Blue R-250 in 40% methanol–10% $CH_3COOH$, and destained in 40% methanol–10% $CH_3COOH$.

*Immunoblotting.* SDS-PAGE gels were blotted at 2 mA $cm^{-2}$ for 1 h to a 0.2 μm pore size nitrocellulose membrane. The nitrocellulose was blocked in 350 mM NaCl, 10 mM tris, pH 7.6, and 2% skimmed milk for 1 h, and then washed three times for 5 min in alkaline phoshatase washing buffer. The nitrocellulose was then incubated for 1 h with rabbit antisera against calreticulin[30] diluted 1 : 1000 in 50 mM tris, 0.15 M NaCl, 0.05% Tween 20, and 0.5% skimmed milk, pH 7.5, then washed three times with alkaline phosphatase washing buffer,

and incubated for 1 h with alkaline phosphatase-conjugated swine immunoglobulins against rabbit IgG diluted 1:4000 in the incubation buffer. This was followed by two 5 min washes in alkaline phosphatase washing buffer, and a 5 min wash in 0.1 M tris, 0.1 M NaCl, and 5 mM $MgCl_2$, pH 9.5. Bound antibodies were detected using alkaline phosphatase development solution (660 µl of 25 mg $ml^{-1}$ 5-bromo-4-chloro-3-indolyl phosphate in *N,N*-dimethylformamide and 660 µl of 50 mg $ml^{-1}$ nitro blue tetrazolium in 70% *N,N*-dimethylformamide mixed with 100 ml 0.1 M tris, 0.1 M NaCl, and 5 mM $MgCl_2$, pH 9.5).

*Two-dimensional gel electrophoresis.* Serum (10 µl) diluted 1:20 with sample buffer was run on immobiline DryStrips and then on 8–18% Excel Gel SDS Gradient gel from Pharmacia Biotech. The first dimension (isoelectric focusing with an immobilized pH gradient) and the second dimension (SDS electrophoresis) were run according to the instructions from Pharmacia. A small change was made in the running conditions for the first dimension: electrophoresis for 1 h at 300 V, 1 mA, followed by 16 h at 1300 V, 1 mA, and finally 1 h at 2500 V, 1 mA.

*Binding of calreticulin to proteins separated by SDS-PAGE or two-dimensional gel electrophoresis.* After electrophoresis the gel was blotted at 1 mA $cm^{-2}$ for 1 h to a 0.45 µm pore size nitrocellulose membrane. The nitrocellulose membrane was blocked in 10 mM Tris, pH 7.3, containing 0.5% Tween 20 for 1 h, and then incubated with biotinylated calreticulin 1:1000 in 0.05 M Tris, 0.01 M $MgCl_2$, and 0.5% Tween 20, pH 7.5, or in 0.01 M Tris, 0.005 M $MgCl_2$, and 0.05% Tween 20, pH 8.5 for 1 h. The membrane was then washed three times for 5 min in the same buffer, after which it was incubated for 1 h with horseradish-peroxidase-conjugated streptavidin 1:5000 in 0.05 M Tris, 0.01 M $MgCl_2$, and 0.5% Tween 20, pH 7.5, or in 0.01 M Tris, 0.005 M $MgCl_2$, and 0.05% Tween 20, pH 8.5, and washed three times for 5 min in the same buffer. Bound calreticulin was detected by means of enhanced chemiluminescence.

*Heat denaturation of proteins.* Protein (OVA, BSA, 1 g) was dissolved in 100 ml phosphate-buffered saline, and then autoclaved at 105 °C for 1 h.

## Results

Calreticulin was purified from the ER fraction of human placental extracts by a combination of temperature-induced phase separation with Triton X-114, ammonium sulfate precipitation and ion exchange FPLC. To ensure that the purified calreticulin did not contain impurities, the fraction from the FPLC containing calreticulin as determined by ELISA with antibodies against the *C*-terminal peptide of calreticulin,[22] was analysed by SDS-PAGE (Fig. 1). Only one band was seen, which in

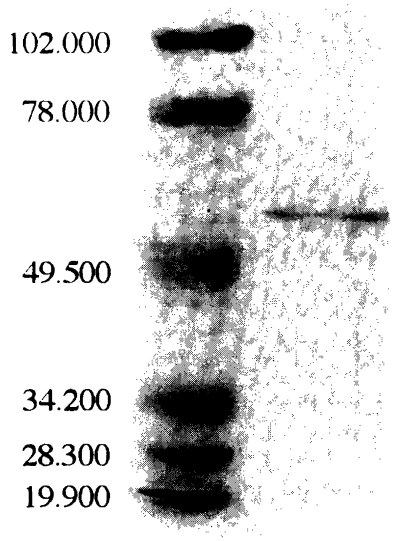


*Fig. 1.* Analysis of purified human placental calreticulin by SDS-PAGE with Coomassie Brilliant Blue staining. (Silver staining of an identical gel showed that calreticulin did not stain, and nor were impurities detected).

immunoblotting reacted with polyclonal rabbit antibodies against the *C*-terminal peptide of calreticulin. The relative molecular weight was estimated to be 60 000, which agrees with the size usually found for calreticulin by SDS-PAGE.[2] To examine whether calreticulin binds mainly to denatured proteins with specific pI values or specific relative molecular weights, a two-dimensional gel blot was made using denatured serum proteins as targets for calreticulin binding. As seen in Fig. 2, human serum contains many proteins that, in denatured form, can interact with calreticulin, and it was found that calreticulin did not prefer to bind proteins with specific pI values or specific relative molecular weights. To test the importance of the relative molecular weight further, calreticulin's interaction with proteins of different size (ubiquitin [8500], RNAse A [13 683], lysozyme [14 300], β-lactoglobulin [17 500], proteinase K [18 500], trypsin [24 000], histone [33 000], pepsin [35 000], OVA [45 000], fetuin [48 700], BSA [68 000], lactoferrin [77 000]) was tested in ELISA format with the proteins coated under denaturing conditions (8 M urea, 50 mM DTT). This also did not show any relationship between the relative molecular weight of a protein and its binding to calreticulin; lysozyme and histone bound very strongly both in their native and denatured form, while the rest of the proteins bound less strongly but equally well (with the strongest binding to the denatured form).

In contrast with its interactions with denatured proteins, calreticulin was found to bind preferentially to basic native proteins. Calreticulin's binding to native proteins was tested as a control in the ELISA format experiments mentioned before and on blots of proteins separated by agarose gel electrophoresis. We found that calreticulin bound only to the basic proteins (lysozyme, lactoferrin, Human IgG/IgM, myeloperoxidase) but not

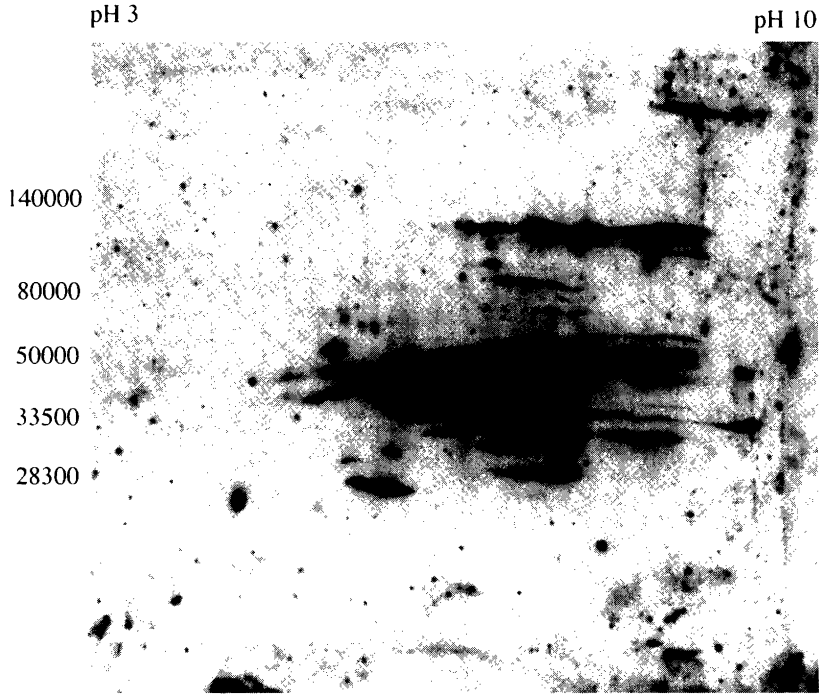


*Fig. 2.* Binding of calreticulin to denatured serum proteins. A sample of human serum was separated by two-dimensional gel electrophoresis, transferred to nitrocellulose and incubated with biotin-labelled calreticulin in in 0.01 M Tris, 0.005 M $MgCl_2$, and 0.05% Tween 20, pH 8.5.

the acidic proteins ($\beta_2$-microglobulin, human serum albumin, proteinase 3) using 0.05 M Tris, 0.01 M $MgCl_2$, and 0.05% Tween 20, pH 8.5 as incubation buffer.

As a consequence of the results above, we decided to investigate in more detail the binding of calreticulin to denatured model proteins with isoelectric points below 7. In ELISA format experiments we observed a larger interaction of calreticulin with denatured BSA and OVA than with the native proteins (Fig. 3). An experiment carried out to test the amount of target protein bound to the well in the ELISA plate under native and denatured coating conditions, showed that the protein coated equally well under native and denatured conditions. Since we found that calreticulin would interact better with denatured proteins than with native, we decided to investigate the influence of the coating conditions in order to determine the relative importance of the state of the substrate and the possible interactions involved (hydrogen bonds, S–S bridges, ionic forces) (Fig. 4). By varying the amount of urea and DTT during coating of substrate, we found that DTT alone did not have any effect on the binding, whereas 8 M urea did have an effect. When both urea and DTT were present, we observed an even larger binding of calreticulin to the denatured proteins. We conclude that breaking the S–S bridges by itself did not have any appreciable effect, but that disturbance of hydrogen bonds and hydrophobic interactions had a large effect on subsequent calreticulin binding. Together urea and DTT had an even larger effect, probably because of the more extensive denaturation of the target proteins under the combined action of

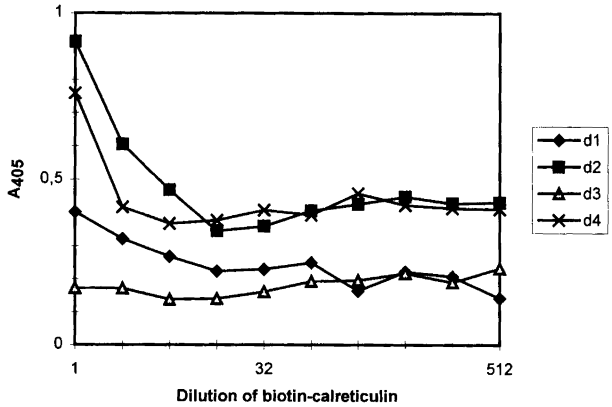


*Fig. 3.* Calreticulin interacts more strongly with denatured BSA and OVA than with the native proteins: (d1) BSA (1 μg $ml^{-1}$) coated from 50 mM sodium carbonate, pH 9.3; (d2) BSA coated from 50 mM sodium carbonate, pH 9.3, 8 M urea, and 10 mM DTT; (d3) OVA (1 μg $ml^{-1}$) coated from 50 mM sodium carbonate, pH 9.3; (d4) OVA coated from 50 mM sodium carbonate, pH 9.3, 8 M urea, and 10 mM DTT. The coatings were followed by incubation with biotinylated calreticulin diluted twofold in 50 mM tris, 10 mM $MgCl_2$, and 0.5% Tween 20, pH 7.5, from a starting concentration of 1 μg $ml^{-1}$.

these two agents. Exactly the same effects of urea and DTT were observed when BSA was first coated from carbonate buffer and then subsequently treated with urea and/or DTT after coating. Thus, the effect of these compounds is not on the coating process itself but on the state of the coated protein.

In these experiments we tested both BSA (a non-

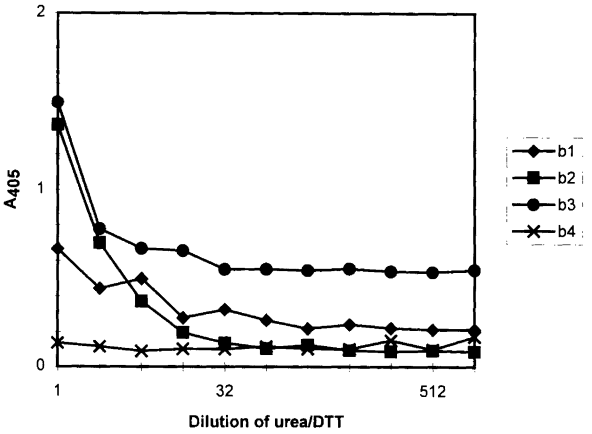


*Fig. 4.* The influence of denaturation of BSA on binding of calreticulin. ELISA plates were incubated with a fixed concentration of biotin-labelled calreticulin after coating of plates with BSA from the following buffers (the components being diluted in the experiment during coating are *italicised.* (b1) 50 mM sodium carbonate, pH 9.3 + *8 M urea;* (b2) 50 mM sodium carbonate, pH 9.3, 0.25 M DTT + *8 M urea;* (b3) 50 mM sodium carbonate, pH 9.3, 8 M urea + *0.25 M DTT;* (b4) 50 mM sodium carbonate, pH 9.3 + *0.25 M DTT.*

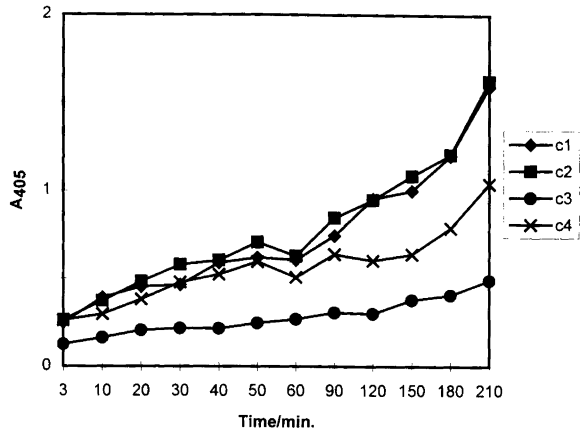


*Fig. 5.* Time-dependent binding of calreticulin to denatured non-glycosylated serum proteins (run through from a Con A column). c1–c4 are different incubation-buffers; (c1) 0.05 M Tris, 0.01 M $MgCl_2$, and 0.5% Tween 20, pH 7; (c2) 0.05 M Tris, 0.01 M $MgCl_2$, 0.5% Tween 20, and 0.01 M mannose, pH 7; (c3) 0.05 M Tris, 0.01 M $MgCl_2$, 0.5% Tween 20, and 0.01 M mannose 1-phosphate, pH 7; (c4) 0.05 M Tris, 0.01 M $MgCl_2$, 0.5% Tween 20, and 0.01 M mannose 6-phosphate, pH 7.

glycosylated protein) and OVA (a glycosylated protein) to see if there was any effect whether or not the protein we analysed for calreticulin binding was glycosylated. The results were identical for both proteins, even though the interaction with OVA was somewhat slower than with BSA. Since denatured BSA and OVA bound equally well to calreticulin, we chose to use BSA in further experiments, because we wanted to be sure that calreticulin did not interact positively or negatively with the carbohydrate part of the substrate.

To determine the nature of the binding in more detail, we examined the time dependency of calreticulin's binding to denatured proteins, and its dependence on pH and ionic composition of the environment. As can be seen in Fig. 5, calreticulin exhibited rather slow time-dependent binding to denatured non-glycosylated serumproteins. Identical results were seen with glycosylated serum proteins and with BSA.

Futhermore, when serum proteins were separated into glycosylated and non-glycosylated proteins by chromatography on Con A Sepharose, no difference in binding of calreticulin was observed using SDS-PAGE-separated proteins transferred to nitrocellulose. The binding of calreticulin to SDS-PAGE-separated serum proteins was not influenced by monosaccharides; however, very high concentrations of 1 M did show some inhibitory effect.

To examine the importance of the pH level, calreticulin's binding to native and denatured BSA was tested in ELISA format (Fig. 6). At pH 4.9 and pH 6.1 we observed a much larger interaction of calreticulin with both the native and denatured BSA than at pH 7.1, pH 8, pH 8.9, and pH 10. Futhermore, we found that at pH 7.1, the interactions were strongly dependent on the presence of divalent cations, and it did not seem to be

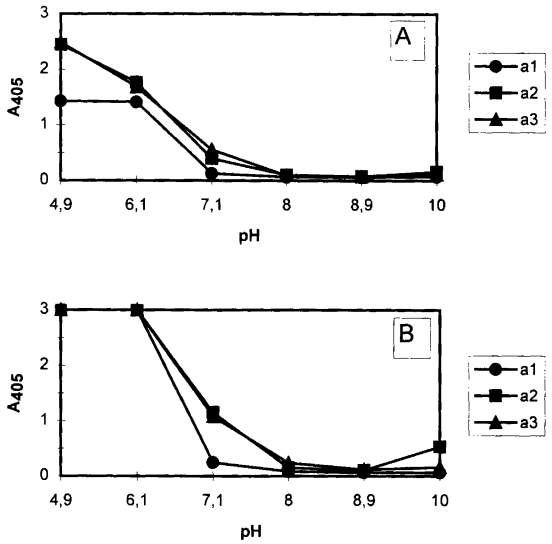


*Fig. 6.* Binding of calreticulin to BSA is dependent on the pH-level and the presence of cations. (A) BSA coated from 50 mM sodium carbonate, pH 7.3 (native conditions); (B) BSA coated from 50 mM sodium carbonate, pH 7.3, 8 M urea, and 50 mM DTT (denaturing conditions). a1–a3 are different incubation buffers; (a1) 50 mM Tris and 0.5% Tween 20; (a2) 50 mM Tris, 0.5% Tween 20, and 10 mM $MgCl_2$; (a3) 50 mM Tris, 0.5% Tween 20, and 10 mM $CaCl_2$.

important which cations were used, as both $Mg^{2+}$ and $Ca^{2+}$ had equal effects. The experiments were carried out in the presence of 10 mM calcium or magnesium to ensure that the number of the ions would not be limiting. Fig. 6 also shows a larger interaction of calreticulin with proteins when the proteins are denatured, especially at the lower pH. This means that near its isoelectric point calreticulin shows optimum chaperone properties. One of the consequences of $Ca^{2+}$ binding to calreticulin at

pH 7 may be to neutralize the negative charge of the chaperone, thus facilitating its interactions with substrates which are themselves negatively charged at pH 7. In fact, this shows directly how the $Ca^{2+}$ concentration may regulate the chaperone activity of calreticulin.

The state of the denatured substrate was found to be important for binding of calreticulin (Fig.7), as no binding to heat-denatured ovalbumin was seen when tested in ELISA format. Heat-denatured ovalbumin could only bind to calreticulin when it had been treated with urea and DTT. From these experiments we conclude that calreticulin preferentially interacts with substrates having a completely unfolded polypeptide chain as in the random coil state or as in newly synthesized nascent polypeptide chains.

In order to determine whether calreticulin would interact differently with non-glycosylated and glycosylated proteins, the experiment from Fig. 5 was repeated with glycosylated proteins (eluted from a Con A column) and with exactly the same results; calreticulin did not discriminate between glycosylated and non-glycosylated serum proteins separated on a Con A column. Glycosylated and non-glycosylated proteins were also compared in ligand blotting experiments in which the incubation time was varied (5, 10, 30 and 60 min) to see whether calreticulin would bind faster to glycosylated than to non-glycosylated proteins. The result showed no difference between 5 min and 60 min incubation; calreticulin bound equally well to glycosylated and non-glycosylated proteins. Moreover, we varied the incubation conditions by adding different monosaccharides to the incubation buffer, to see whether the presence of carbohydrates would influence the binding of calreticulin to denatured proteins. In these experiments we found that mannose had no effect, but mannose 6-phosphate, and mannose 1-phosphate, inhibited binding between calreticulin and denatured proteins at pH 7 (Fig. 5). We also tested calreticulin's binding to denatured BSA with 12 other carbohydrates, including glucose, and found that they were without effect on the binding.

As a control on the influence of the phosphorylated sugars, we tested other phosphate-containing compounds (phosphorylcholine chloride, myoinositol, myoinositol 1,4,5-trisphosphate, phosphotyrosine, phosphoserine, phosphothreonine, phosphoethanolamine, ATP), and found that they all inhibited calreticulin binding to denatured BSA at pH 7. Thus, the mere presence of a phosphate had a restraining effect on calreticulin binding to denatured proteins. The nature of this inhibition may involve interaction between the phosphates and $Ca^{2+}$.

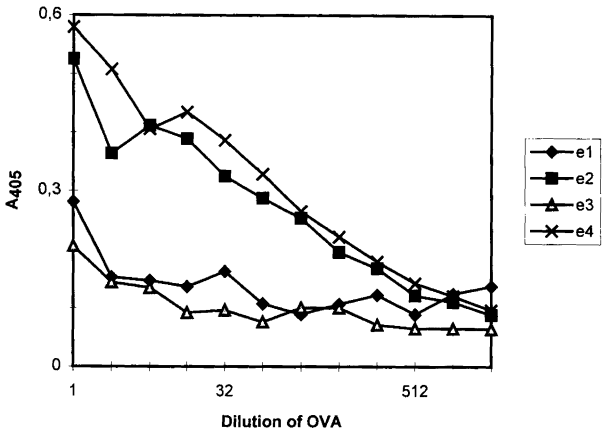


*Fig. 7.* The state of the denatured substrate is important for binding of calreticulin. ELISA plates were coated with OVA (1 μg $ml^{-1}$) or heat denatured OVA (1 μg $ml^{-1}$) from the following buffers (the component being diluted is *italicised*) (e1) *OVA* coated from 50 mM sodium carbonate, pH 9.3; (e2) *OVA* coated from 50 mM sodium carbonate, pH 9.8, 8 M urea, and 50 mM DTT; (e3) *heat-denatured OVA* coated from 50 mM sodium carbonate, pH 9.3; (e4) *heat-denatured OVA* coated from 50 mM sodium carbonate, pH 9.3, 8 M urea, and 50 mM DTT.

## Discussion

Chaperones associate with folding intermediates of proteins and promote their efficient folding while preventing their degradation.[31] An important property of a chaperone is therefore differential binding to denatured versus native proteins. In the present work we have investigated the binding of calreticulin to native and denatured proteins. We found that calreticulin interacted more strongly with denatured proteins than with native proteins, which agrees with calreticulin being a chaperone. We found that breaking the S–S bridges in a target molecule was not sufficient for calreticulin to bind; however, denaturation by breaking the hydrogen bonds and hydrophobic interactions increased binding of calreticulin. When the hydrogen bonds, hydrophobic interactions, and the S–S-bridges had all been broken, even greater binding was observed. This can be explained by a stronger denaturing effect of the combined action of urea and DTT on the protein substrate.

Calreticulin binding to denatured proteins was found to be time-dependent and rather slow. The pH is also very important. We found that if the pH value was below 7 calreticulin would interact more strongly with the proteins (presumably because of the acidic nature of calreticulin), but at pH 7 the interactions were dependent on the presence of divalent cations (either $Ca^{2+}$ or $Mg^{2+}$). At pH values above 8, virtually no interactions were seen. Presumably, the divalent cations neutralize the negative charges on calreticulin, making it easier for calreticulin to interact with a protein. We conclude that when calreticulin binds to acidic proteins at physiological pH, the binding is cation-dependent. We found no relationship between the relative molecular weight of a protein and its interaction with calreticulin. We conclude that the relative molecular weight of a protein has no influence on whether calreticulin will bind to the denatured form of the protein or not. When we investigated calreticulin binding to native proteins, after agarose gel electrophoresis, we saw a decreasing tendency towards calreticulin binding when the salt concentration was

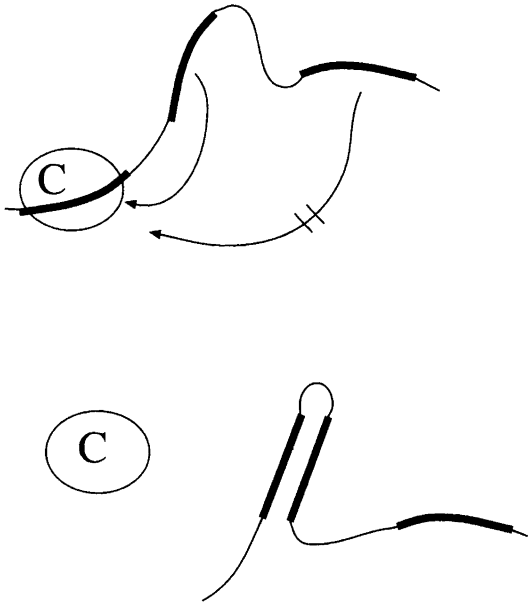


*Fig. 8.* Model of the involvement of calreticulin in protein folding. 'C' is calreticulin, and the thick lines are β-strands. Calreticulin binds to a hydrophobic β-strand region of the unfolded protein, but can be displaced when the correct β-strand binds. Another β-strand, which would impair correct protein folding, binds too weakly to be able to displace calreticulin. When calreticulin is displaced it can bind to another hydrophobic part of the protein promoting correct protein folding in that area.

increased. This is in agreement with previous results[32] and suggest that the interactions between calreticulin and a protein are partly ionic. We also found that calreticulin bound to native basic proteins under some conditions, but not the native acidic proteins. This is most likely due to the negative charge of calreticulin. Also in agreement with this was the finding that phosphorylated compounds inhibited the interaction between calreticulin and denatured proteins, presumably by binding to the divalent cations present on calreticulin, thereby modulating the effective charge of calreticulin. It is not known whether this is relevant to calreticulin's functions *in vivo*, or if there is a mechanism that prevents this.

It is a possibility that calreticulin, because of its lectin properties, would bind faster to glycosylated than to non-glycosylated proteins, or that the presence of fully trimmed carbohydrate trees would interfere with the binding of calreticulin. As published for calnexin by Ware *et al.*[33] calreticulin could make an initial, fast, contact via the glycan moiety, and then later a more stable contact with the surface of the incompletely folded protein. We therefore separated human serum proteins into glycosylated and non-glycosylated proteins on a Con A column. We found that calreticulin interacted equally well with both the denatured glycosylated and the denatured non-glycosylated proteins. Moreover attempts to stimulate calreticulin binding to denatured BSA by adding different carbohydrates to the incubation buffer did not seem to have any influence. We did find, though, that carbohydrates in large concentrations (0.1–1 M) could have an effect (either stimulatory or inhibitory), but at more relevant physiological concentrations carbohydrates did not have any effect.

We conclude that calreticulin has the ability to bind hydrophobic sequences in fully unfolded proteins. As shown in Fig. 8 this may promote protein folding by a passive mechanism whereby calreticulin binds with an affinity which requires a relatively strong displacing force that may be delivered by stabilizing β-strand interactions or similar appropriate folding intermediate interactions.

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