**Ca^{2+} Regulation of Interactions between Endoplasmic Reticulum Chaperones**

(Received for publication, July 9, 1998, and in revised form, December 17, 1998)

Elaine F. Corbett‡‡, Kim Oikawa‡, Patrice Francoise§, Daniel C. Tessier‡, Cyril Kay§, John J. M. Bergeron**, David Y. Thomas§, Karl-Heinz Krause†, and Marek Michalak‡‡

From the ‡‡Medical Research Council of Canada (MRC) Group in Molecular Biology of Membranes, the MRC Group in Protein Structure and Function, Protein Engineering Network of Centers of Excellence and the Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2H7, Canada, and the ‡Division of Infectious Diseases, University Hospital, CH-1216 Geneva, Switzerland, the |Genetics Group, Biotechnology Research Institute, National Research Council of Canada, Montreal H4P 2R2, and the ||Department of Anatomy and Cell Biology, McGill University, Montreal, Quebec H3A 2B2, Canada.

Cascade Blue (CB), a fluorescent dye, was used to investigate the dynamics of interactions between endoplasmic reticulum (ER) luminal chaperones including calreticulin, protein disulfide isomerase (PDI), and ERp57. PDI and ERp57 were labeled with CB, and subsequently, we show that the fluorescence intensity of the CB-conjugated proteins changes upon exposure to microenvironments of a different polarity. CD analysis of the purified proteins revealed that changes in the fluorescence intensity of CB-ERp57 and CB-PDI correspond to conformational changes in the proteins. Using this technique we demonstrate that PDI interacts with calreticulin at low Ca^{2+} concentration (below 100 μM), whereas the protein complex dissociates at >400 μM Ca^{2+}. These are the Ca^{2+} concentrations reminiscent of Ca^{2+} levels found in empty or full ER Ca^{2+} stores. The N-domain of calreticulin interacts with PDI, but Ca^{2+} binding to the C-domain of the protein is responsible for Ca^{2+} sensitivity of the interaction. ERp57 also interacts with calreticulin through the N-domain of the protein. Initial interaction between these proteins is Ca^{2+}-independent, but it is modulated by Ca^{2+} binding to the C-domain of calreticulin. We conclude that changes in ER luminal Ca^{2+} concentration may be responsible for the regulation of protein-protein interactions. Calreticulin may play a role of Ca^{2+} “sensor” for ER chaperones via regulation of Ca^{2+}-dependent formation and maintenance of structural and functional complexes between different proteins involved in a variety of steps during protein synthesis, folding, and post-translational modification.

Calreticulin is a ubiquitous and highly conserved Ca^{2+}-binding, resident protein of the endoplasmic reticulum (ER)1 membranes (1). The protein has been implicated to play a role in a variety of cellular functions including Ca^{2+} storage and signaling, regulation of gene expression, cell adhesion, and autoimmunity while also serving as a lectin-like chaperone (2–10). Calreticulin can be divided into three regions: a highly conserved N-domain, the proline rich P-domain, containing a high affinity Cu^{2+}-binding site and lectin-like function, and the C-domain that contains the high capacity, low affinity Cu^{2+}-binding (storage) site (11–14). The P-domain of calreticulin shares amino acid sequence identity with calnexin, an integral ER membrane chaperone (15). Calnexin and calreticulin are both lectins, which recognize and bind to N-glycans in the form of GlcNAc_{2}Man_{n}Glc_{3} (9, 14). Helenius and co-workers (9) proposed that the lectin-like activity of calreticulin and calnexin plays a critical role in quality control process during protein synthesis and folding.

In our earlier studies (1, 16) we have identified calreticulin as one of the major Ca^{2+}-binding, multifunctional proteins of the ER membrane, and we documented that the protein interacts with PDI. PDI is an abundant, ER luminal protein that catalyzes a variety of thiol/disulfide exchange reactions (17, 18). ERp57 is another ER chaperone, a homologue of PDI, with thiol-dependent reductase (19) and cysteine-dependent protease activities (20). Recently, High and co-workers (21, 22) reported that ERp57 can be cross-linked to monoglucosylated glycoproteins that are substrates for calnexin and calreticulin suggesting that ERp57 may also be a lectin-like chaperone. Zapun et al. (23) identified functional complexes between ERp57, calreticulin, and calnexin and showed that disulfide isomerase activity of ERp57 is much greater in the presence of calreticulin or calnexin suggesting a functional association between these proteins. Direct interaction between calreticulin and ERp57 has not yet been reported.

In addition to their chaperone function, the majority of ER resident proteins, including calreticulin and PDI, bind Ca^{2+} and Zn^{2+} and contribute to the Ca^{2+} storage capacity of the ER and cellular Ca^{2+} homeostasis (10, 13, 16, 24–33). Therefore, changes in the luminal Ca^{2+} concentration due to Ca^{2+} release via inositol trisphosphate receptor/ryanodine receptor and Ca^{2+} uptake via Ca^{2+}-ATPase (SERCA) (34) are expected to play an important role in the control of chaperoning and other functions of these proteins (13, 32). How chaperones interact to facilitate protein folding and what is a role of the ER luminal ions in these processes is not known.

In this paper we studied interactions between calreticulin and two related ER membrane chaperones, PDI and ERp57, linepropanesulfonic acid; DTPA, diethylenetriaminepentaacetic acid; SERCA, sarco/plasmic/endoplasmic reticulum Ca^{2+}-ATPase.

*This work was supported by grants from the Medical Research Council of Canada (to M. M., J. J. M. B., and D. Y. T.), from the Swiss National Foundation (to K. H. K.), and from the Protein Structure and Function, Protein Engineering Network of Centers of Excellence and the Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2H7, Canada, the |Genetics Group, Biotechnology Research Institute, National Research Council of Canada, Montreal H4P 2R2, and the ||Department of Anatomy and Cell Biology, McGill University, Montreal, Quebec H3A 2B2, Canada.

‡‡ Medical Research Council of Canada Senior Scientist and Medical Scientist of the Alberta Heritage Foundation for Medical Research. To whom correspondence should be addressed: Dept. of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2H7, Canada. Tel.: 780-492-2256. Fax: 780-492-0986; E-mail: marek.michalak@ualberta.ca.

1 The abbreviations used are: ER, endoplasmic reticulum; PDI, protein disulfide isomerase; CB, Cascade Blue; PBS, phosphate-buffered saline; Pipes, 1,4-piperazinediethanesulfonic acid; Mops, 4-morpho-
and we investigated the role of Ca²⁺ in these protein-protein interactions. We show that calreticulin interacts with PDI in a Ca²⁺-dependent manner reminiscent of emptying and refilling of the ER Ca²⁺ stores. Formation of the ERp57-calreticulin complex was initiated by a Ca²⁺-independent conformational change in ERp57 followed by additional Ca²⁺-dependent conformational changes in the complex. Ca²⁺ sensitivity of the calreticulin-PDI and calreticulin-ERp57 complexes was confined to the high capacity Ca²⁺ binding, C-domain of calreticulin. We conclude that calreticulin interacts with ER luminal chaperones and that the protein may play a role of Ca²⁺ "sensor" for these interactions.

**EXPERIMENTAL PROCEDURES**

**Materials**—The FluoroTag FITC Conjugation Kit, Mops, Pipes, DTPA, RNase, and EGTA were obtained from Sigma. Dithiothreitol was purchased from ICN Biomedicals, Inc. Cascade Blue acetyl azide (catalog number C-2284) was from Molecular Probes, Inc. QuixSep microdialyzer was obtained from Membrane Filtration Products, Inc. Sephacryl G-25M was from Amersham Pharmacia Biotech. Fresh canine pancreas was obtained from the Surgical Medical Research Institute at the University of Alberta. All chemicals were of the highest grade available.

**Circular Dichroism**—Calreticulin, and ERp57 were purified by ammonium sulfate precipitation procedures as described previously (35, 36). The recombinant domains of calreticulin and recombinant glutathione S-transferase were expressed in Escherichia coli and purified (11, 36). Protein disulfide isomerase (PDI) was isolated from bovine liver by a procedure of Lambert and Freedman (37) and modified as described by Baksh et al. (16). Human ERp57 was expressed in E. coli and purified (23).

**Fluorescence Labeling**—Proteins were labeled with Cascade Blue (CB) acetyl azide by adaptation to a FluoroTag FITC Conjugation Kit procedure as recommended by the manufacturer. Briefly, CB (1.11 mg/ml) was dissolved in 100 mM carbonate/bicarbonate buffer, pH 9.0. Six hundred μg of purified PDI or ERp57 (2.6 mg/ml) in a 100 mM sodium carbonate/bicarbonate buffer, pH 9.0, was used directly for labeling. The dye was added dropwise to the protein mixture with constant stirring. The reaction vial was incubated in the dark for 2 h at room temperature with gentle stirring. Labeled proteins were separated from the free dye on a Sephacryl G-25M column (3.5 ml, bed height 2.6 cm) previously equilibrated with PBS. The reaction mixture was applied, and fractions (0.25 ml each) were eluted with PBS. The fluorescence of each fraction was determined at the excitation wavelength 385 nm and the emission wavelength 430 nm using a Perkin-Elmer spectrophotometer. Fractions containing labeled protein were combined and used directly for protein-protein interaction studies. Determination of the stoichiometry of the labeling revealed that there were 4 and 5 molecules of CB conjugated per each molecule of ERp57 and PDI, respectively, indicating that over 90% of the protein was labeled with the dye.

**Fluorescence Measurements**—Fluorescence measurements were performed at an excitation wavelength 385 nm (slit, 15 nm) and an emission wavelength 430 nm (slit, 15 nm) at room temperature using a Luminescence Spectrometer LS50B (Perkin-Elmer). Fluorescence intensities were measured with constant stirring in 1.5 ml of a binding buffer containing 10 mM Mops, pH 7.0, 100 mM KCl, 2 mM MgCl₂, 0.5 mM EGTA. Appropriate proteins and/or ions were added to the reaction mixture, and the corresponding changes in fluorescence intensity were monitored. Quantum yields of CB or CB-labeled proteins were calculated from the emission spectra (420–460 nm) obtained at the excitation wavelength 385 nm. Initial rates of the protein-protein interaction and time constants were calculated using an exponential decay function using Origin version 4.1 software.

**Circular Dichroism Measurements**—For circular dichroism (CD) analysis, proteins were dialyzed for 16 h against a buffer containing 25 mM Pipes, pH 6.8, 100 mM NaCl, 1 mM EGTA, 1 mM dithiothreitol. Dialysis was performed using Spectra/Por dialysis tubing (cut-off 12–14 kDa) in a QuixSep Microdialyzer. CD measurements were carried out on a Jasco J-720 spectropolarimeter (Jasco Inc., Easton, MD), interfaced to an Epson Equity 386/25 and controlled by Jasco software. The thermostable cell holder was maintained at 25 °C with a Lauda RMS circular water bath (Lauda, Westbury, NY). The instrument was routinely calibrated with ammonium d(+)-10-camphor sulfoxide at 290.5 and 192 nm. Each sample was scanned 10 times, and noise reduction was applied to remove the high frequency before calculating molar ellipticities. The voltage to multiplier was kept below 500 V to prevent distortion of the CD spectrum. The cell path length used was 0.02 cm, and the protein concentrations were 0.6 mM in the far ultraviolet. Concentrations were determined on a Cary 3 UV-visible spectrophotometer and were corrected for light scattering. Molar extinction coefficients were calculated from tyrosine and tryptophan compositional values and were 43,780, 45,040, and 81,480 for ERp57, PDI, and calreticulin, respectively. Molar ellipticities were calculated from the following equation: \[ \theta = \frac{\lambda l c}{n} \times 1 \times c, \] where \( \theta \) is in millidegrees, \( l \) is the pathlength in centimeters, and \( c \) is the concentration in moles/liter \( \times \) number of amino acids in the sequence. The unit for molar ellipticity is degree centimeter squared per pmol. The CD spectra were analyzed for secondary structure elements by the Contin ridge regression analysis program of Provencher and Glowacki (38).

**Miscellaneous**—All recombinant techniques were conducted according to standard protocols (39). Refolding of RNase B in the presence of CB-labeled proteins was carried out as described previously (23). Free Ca²⁺ concentrations were calculated using Max Chelator, Winmax version 1.70.

**RESULTS**

**Changes in Fluorescent Intensity of Cascade Blue (CB) Are Indicative of Conformational Changes in PDI and ERp57—CB acetyl azide reacts with aliphatic amines in proteins to yield stable carboxamides, is highly fluorescent, and resists quenching upon protein conjugation (40). One unique feature of CB is that it has a different fluorescence intensity depending on the polarity of the solvent used (Fig. 1 and Table I). For example, CB alone had relatively high fluorescence intensity in PBS and water but significantly lower fluorescence in nonpolar solvents such as n-propyl alcohol and n-butanol (Fig. 1 and Table I). This observation suggested to us that the dye may also be sensitive to exposure to the hydrophilic or hydrophobic environments in a protein. To test these ER luminal chaperones, PDI and ERp57 were labeled with CB to generate CB-PDI and CB-ERp57, respectively. Labeled proteins displayed emission and excitation spectrum similar to the unconjugated fluorophore. Similar to CB alone, fluorescence intensity of the protein-conjugated CB (CB-ERp57 and CB-PDI) had different relative quantum yields in various solvents (Table I) suggesting that the protein-conjugated dye had a different fluorescence intensity depending on whether it was exposed to a polar or nonpolar microenvironment.
Native PDI and ERp57 catalyze a variety of thiol/disulfide exchange reactions (17–19, 23), and their chaperone activity can be estimated by their ability to refold RNase B (23). Furthermore, ERp57 disulfide isomerase activity is increased in the presence of calreticulin or calnexin (23). It was, therefore, important to demonstrate that CB labeling of PDI and ERp57 does not interfere with the function of these chaperones. We examined disulfide isomerase activity of CB-PDI and CB-ERp57 on the refolding of RNase B the presence and absence of calreticulin and calnexin (Fig. 2). Fig. 2 shows that similar to native PDI and ERp57 (23), CB-PDI and CB-ERp57 catalyzed refolding of RNase B indicating that labeled proteins retained their chaperone activity. Refolding of RNase B catalyzed by CB-PDI or CB-ERp57 was not influenced by changes of Ca$^{2+}$ or Zn$^{2+}$ concentration.

Next we tested effects of ions (Ca$^{2+}$ and Zn$^{2+}$) and purified calreticulin on CB alone and on CB labeled proteins. Fluorescence intensity of CB alone did not change in the presence of Ca$^{2+}$, Zn$^{2+}$, or purified calreticulin (Fig. 3A). However, the amplitude of fluorescence of the labeled proteins was sensitive to ion-induced conformational changes. Fig. 3, B and C show that addition of Zn$^{2+}$ to CB-PDI or CB-ERp57 resulted in significant decrease in fluorescence intensity of the labeled proteins. To test whether changes in fluorescence intensity induced by Zn$^{2+}$ (Fig. 3, B and C) reflected conformational alterations in CB-PDI and CB-ERp57 we carried out CD analysis of the purified proteins. Fig. 4 reveals that the CD spectra of ERp57 and PDI are very similar in shape, having minima at 219 and 210 nm with molar ellipticity values of $-10330\deg$ and $-10180\deg$ at 219 nm and $-9490\deg$ and $-9670\deg$ at 210 nm, respectively. The Contin version program for calculating secondary structural elements (Table II) indicates the $\alpha$-helical content for both proteins was $25$–$30\%$, and the combined $\beta$-sheet and $\beta$-turn was $-50\%$. The values for PDI are similar to those reported in previous studies by Wetterau et al. (41, 42). Both proteins underwent a conformational change upon addition of Zn$^{2+}$ with a loss in the amount of $\alpha$-helix and a concomitant increase in $\beta$-sheet-$\beta$-turn. Similarly, pancreatic calreticulin showed no change upon addition of Ca$^{2+}$ but underwent a reduction in $\alpha$-helix and an increase in combined $\beta$-sheet-$\beta$-turn upon addition of 2 mM Zn$^{2+}$, a finding also reported earlier by Khanna et al. (26). The apoprotein has $-10\%$ $\alpha$-helix and $50\%$ $\beta$-sheet-$\beta$-turn, upon analysis (Table II). We concluded that Zn$^{2+}$-dependent changes in the fluorescence intensity of CB-ERp57 and CB-PDI (Fig. 3) may be due to conformational changes in the proteins (Fig. 4) resulting in exposure of the conjugated CB to protein microenvironments of a different polarity (Fig. 1).

**Interactions between Calreticulin and PDI Are Regulated by Ca$^{2+}$**—Can the observed conformational changes in the CB-PDI and CB-ERp57 be applied to study protein-protein interactions? We first investigated the interaction between CB-PDI and calreticulin (Fig. 5). CB-PDI was incubated with Zn$^{2+}$ to induce conformational changes in the protein followed by addition of the purified pancreatic calreticulin (Fig. 5A). Fig. 5A shows that calreticulin induced a very rapid increase in fluorescence intensity of CB-PDI indicative of protein-protein interaction-induced conformational changes in the protein. The calculated initial rate of calreticulin and PDI interaction was $21.4 \pm 0.6$ units/s (mean $\pm$ S.E., $n = 3$), whereas the time constant of the process was $0.16 \pm 0.01$ s (mean $\pm$ S.E., $n = 3$). Fig. 5B illustrates that the effect was saturable with respect to calreticulin and did not require the presence of Zn$^{2+}$. Identical results were obtained with E. coli- and Pichia-expressed recombinant proteins (data not shown). Fig. 5C reveals that, in agreement with our earlier observations (16), calreticulin did not interact with PDI in the presence of 1 mM Ca$^{2+}$. Hormonestimulated Ca$^{2+}$ depletion results in lowering of the ER luminal free Ca$^{2+}$ concentration below 100 $\mu$M (32, 43, 44). We have tested, therefore, if calreticulin and CB-PDI will interact under the conditions of Ca$^{2+}$ store depletion. Addition of EGTA to lower free Ca$^{2+}$ concentration to 50 $\mu$M restored interaction between calreticulin and CB-PDI as revealed by changes in the

**TABLE I**

| Solvent         | CB   | CB-ERp57 | CB-PDI |
|-----------------|------|----------|--------|
|                 | $Q$  | $\lambda_{max}$ | $Q$  |
| PBS             | 1    | 416      | 1      |
| Water           | 0.94 | 416      | 0.83   |
| Ethanol         | 0.68 | 408      | 0.63   |
| Methanol        | 0.62 | 407      | 0.16   |
| 50% glycerol    | 0.38 | 415      | 0.92   |
| 25% glycerol    | 0.28 | 415      | 0.19   |
| Propyl alcohol  | 0.005| 412      | 0.34   |
| Butanol         | 0.0037| 413    | 0.22  |

**FIG. 2.** Refolding of RNase B catalyzed by CB-PDI and CB-ERp57. Refolding of reduced RNase B was carried out in the presence of CB-ERp57 (ERp57), CB-PDI (PDI), or without the catalyst (CTL) and in the presence of calnexin (CNX) or calreticulin (CRT) by the method described previously (23). The reaction was terminated after indicated times, and the RNase B conformation was examined by non-denaturing polyacrylamide gel electrophoresis. Unfolded RNase B has the slowest mobility (U), and the native form (N) has the greatest mobility.

**FIG. 3.** Zn$^{2+}$-induced conformational changes in Cascade Blue-labeled PDI and ERp57. CB labeling of PDI and ERp57 was carried out as described under “Experimental Procedures.” Changes in the fluorescence intensities of CB (A) and CB-PDI (B) and ERp57 (C) were monitored in the absence or presence of 500 $\mu$M Zn$^{2+}$ (A and B), and 1 mM Ca$^{2+}$ (C). A, the effects of Zn$^{2+}$; B, 1 mM Ca$^{2+}$; and purified pancreatic calreticulin on fluorescence intensity of CB alone was examined. B and C, effects of 500 $\mu$M Zn$^{2+}$ on CB-PDI and CB-ERp57. Bars represent the duration of incubation with ions or purified proteins. Arrowsheads depict the time of addition.
fluorescence intensity of the CB-labeled protein (Fig. 5C). Readdition of Ca$^{2+}$ (to 1 mM) rapidly reduced the fluorescence to the original level indicative of dissociation of the protein complex (Fig. 5C). Ca$^{2+}$ titration experiments revealed that the EC$_{50}$ for Ca$^{2+}$ for calreticulin-PDI dissociation was approximately 110 ± 15 μM (mean ± S.E., n = 3). Thus, there is a saturable, rapidly reversible and Ca$^{2+}$-dependent (under physiologically relevant Ca$^{2+}$ concentrations) interaction between calreticulin and PDI.

Interactions between Calreticulin and ERp57—High and coworkers (21, 22) have shown that ERp57, similar to calreticulin and calnexin, is involved in chaperoning of glycoproteins. Furthermore, Zapun et al. (23) reported functional interactions between calreticulin and ERp57. However, direct interaction between calreticulin and ERp57 has not yet been documented. We utilized CB-ERp57 to investigate binding of calreticulin to ERp57 and the role of Ca$^{2+}$ in these protein-protein interactions. Fig. 6A shows that addition of calreticulin to CB-ERp57 either in the presence or absence of Ca$^{2+}$ had no effect on fluorescence intensity of CB-ERp57, suggesting that under these conditions the proteins may have not interacted. Since Zn$^{2+}$ induced conformational changes in ERp57 (Fig. 3), we tested if the presence of Zn$^{2+}$ had any effect on the protein interaction with calreticulin. Zn$^{2+}$ induced a decrease in fluorescence intensity of CB-ERp57 alone (Fig. 3). In contrast, addition of calreticulin to CB-ERp57 in the presence of Zn$^{2+}$ resulted in an increase in the fluorescence intensity (Fig. 6A) indicating that conformational changes in ERp57 were required to initiate its interaction with calreticulin. Chelating of Zn$^{2+}$ with DTPA resulted in a decrease in the intensity of fluorescence, indicative of dissociation of the protein complex (Fig. 6A). The initial rate of this interaction was 23.4 ± 0.4 units/s (mean ± S.E., n = 3), and it was similar to that observed for calreticulin and PDI interaction (21.4 ± 0.6 units/s; mean ± S.E., n = 3). The time constant of interaction between ERp57 and calreticulin (1.4 ± 0.1 s; mean ± S.E., n = 3) was approximately 9-fold higher than between PDI and calreticulin. Fig. 6B illustrates that changes in the fluorescence intensity of CB-ERp57 were saturable with respect to calreticulin. Initial interactions between ERp57 and calreticulin were not affected by Ca$^{2+}$ (Fig. 6A). Surprisingly, Ca$^{2+}$ had an effect on already formed calreticulin-ERp57 protein complex. Fluorescence intensity of CB-ERp57 and calreticulin complex was increased by addition of 1 mM Ca$^{2+}$ (Fig. 6B). Titration of calreticulin binding to ERp57 in the presence or absence of Ca$^{2+}$ revealed that Ca$^{2+}$ had no effect on the calreticulin affinity to bind ERp57 but only on the fluorescence intensity of the complex. We concluded that Ca$^{2+}$ induced new conformational change in the calreticulin-ERp57 complex without recruiting additional molecules of calreticulin. The Ca$^{2+}$-dependent en-

![Fig. 4. CD analysis of ER luminal proteins. CD spectra of purified calreticulin, PDI, and recombinant ERp57 were carried out as described under “Experimental Procedures.” The data are plotted as molar ellipticity versus wavelength for the proteins in the absence (—) and presence of Zn$^{2+}$ (- - -).](image-url)
hancement of fluorescence was reversed by addition of EGTA (Fig. 6B). Analysis of the Ca$^{2+}$ dependence of this process revealed that the EC$_{50}$ for Ca$^{2+}$ was approximately 400 ± 30 μM (mean ± S.E., n = 3). Thus, interactions between calreticulin and ERP57 initially required a Zn$^{2+}$-dependent but Ca$^{2+}$-independent conformational change in the ERP57. The properties of the complex were further influenced by increased Ca$^{2+}$ concentration.

**Interaction between Calreticulin and PDI and ERP57 Induces Conformational Changes in Protein Complexes**—We used CD analysis to further investigate interaction between calreticulin and ERP57 and PDI. Fig. 7 represents the observed and calculated CD spectra of complexes between calreticulin and ERP57 and PDI. Their secondary structural analysis is indicated in Table II. In the absence of Ca$^{2+}$ and Zn$^{2+}$ the ERP57 complex with calreticulin showed comparable observed and calculated spectra, the latter estimated by weight percentage of each component of the complex. This would imply that under these conditions there was an interaction with a minimal attendant conformational change. In agreement with our fluorescence measurements, addition of Zn$^{2+}$ to the mixture resulted in a larger change in the observed versus calculated spectra (Fig. 7 and Table II). The PDI complex with calreticulin produced small changes for the observed apo and Ca$^{2+}$ conditions relative to the calculated values, whereas Zn$^{2+}$ once again produced the greatest change. Ca$^{2+}$ resulted in a slight increase in α-helix. The apo and Zn$^{2+}$ conditions showed a drop in α-helix upon Conit analysis.

**A Role of Calreticulin Domains in Protein-Protein Interactions**—In order to establish the specific region of calreticulin involved in the interaction with PDI and ERP57, we expressed calreticulin domains in *E. coli*. The following domains of calreticulin were used: the N-domain (the NH$_2$-terminal 182 amino acids of the protein), the P-domain (residues 182–273), the N + P-domain (residues 1–273), and the C-domain (residues 270–401) (11). Previously we showed by ligand blotting, affinity chromatography, and the yeast two-hybrid system that PDI interacts with the N-domain and P-domain of calreticulin in the absence of Ca$^{2+}$ (16). Fig. 8 shows that in the absence of Ca$^{2+}$ the P- and C-domains of calreticulin had no effect on the intensity of fluorescence of CB-PDI, suggesting that under those conditions these domains did not interact with calreticulin. Addition of the N-domain or N + P-domain of calreticulin to CB-PDI in the presence of 50 μM Ca$^{2+}$ induced changes in the fluorescence intensity of the CB-PDI indicative of protein-protein interactions similar to those observed in the presence of full-length calreticulin (compare Fig. 8C and Fig. 5C). Increasing Ca$^{2+}$ concentration up to 1 mM had no effect on fluorescence intensity of the CB-PDI and N-domain (Fig. 8D) or CB-PDI and N + P-domain complexes (Fig. 8C). This is in contrast to the Ca$^{2+}$-dependent dissociation of the full-length calreticulin and CB-PDI complex (Fig. 5). We concluded that Ca$^{2+}$ binding to the C-domain of calreticulin was responsible for Ca$^{2+}$-dependent dissociation of the calreticulin-PDI complex. Thus, the N-domain of calreticulin is required and sufficient for interaction between calreticulin and PDI. The C-domain of the protein plays a role in dissociation of the protein complex.

Next we examined the role of calreticulin domains in interactions with ERP57. Fig. 8, E and F, shows that the P- and C-domain of calreticulin did not have any effect on the fluorescence intensity of CB-ERP57 regardless of the conditions used. However, addition of the N-domain or N + P-domain of the protein resulted in an increase in the intensity of fluorescence of CB-ERP57 suggesting that the two proteins interacted (Fig. 8, G and H). The effect of the N-domain or N + P-domain was identical to that of the full-length calreticulin (compare Fig. 8, G and H, and Fig. 5). Since the P-domain alone did not have any effect on the CB-ERP57 fluorescence, we concluded that the N-domain of calreticulin interacted with ERP57. Similar to the full-length calreticulin, initial interaction between the N-domain of calreticulin and CB-ERP57 was Ca$^{2+}$-independent and required the presence of Zn$^{2+}$ (Fig. 8, G and H). Importantly,
The N-domain of calreticulin is essential for interaction between calreticulin and ERp57. The P-domain does not participate in the interaction. Once again these results suggest that the C-domain of the protein plays a role in Ca\(^{2+}\)-dependent augmentation of the calreticulin-ERp57 complex.

**DISCUSSION**

In this report we utilized CB, a fluorescent dye, to investigate the dynamics of the interactions between calreticulin and two related ER membrane chaperones, PDI and ERp57. These interactions are modulated by changes in protein conformation and by fluctuations in free Ca\(^{2+}\) concentrations reminiscent of emptying and refilling of the ER Ca\(^{2+}\) stores. Calreticulin interacts with PDI at low Ca\(^{2+}\) concentration (<100 \(\mu\)M), but the protein complex dissociates upon increased Ca\(^{2+}\) concentration (>500 \(\mu\)M). Formation of the ERp57-calreticulin complex was initiated by a Ca\(^{2+}\)-independent conformational change in ERp57 followed by a Ca\(^{2+}\)-dependent conformational change in the complex. Ca\(^{2+}\) binding to the C-domain of calreticulin is responsible for the dissociation of the calreticulin-PDI complex and for the modulation of the interaction between calreticulin and ERp57, suggesting that this domain of the protein may play a role of a Ca\(^{2+}\) sensor for ER membrane chaperones. These results suggest changes in the free ER luminal Ca\(^{2+}\) concentration may be important for the regulation of these chaperone-chaperone interactions.

CB has been used previously as a fluorescent indicator for labeling of protein, including antibodies (40). However, to our knowledge, this is the first time the dye has been utilized to study changes in protein conformation due to either ion binding or protein-protein interactions. This property of the dye is likely a result of different behaviors of CB in solvents (environments) of various polarities. Indeed, we show that the fluorescence intensity of CB changes with the exposure to hydrophobic or hydrophilic environments suggesting that exposure of the dye to different regions in proteins may also lead to changes in its fluorescence intensity. For example, changes in fluorescence intensity of CB-PDI and CB-ERp57 due to Zn\(^{2+}\) binding or interaction with calreticulin correspond with changes in their CD spectra. It is conceivable that the dye may be used to study other protein-protein interactions involving conformational changes in proteins.

One of the most important observations in this study is that the interactions between calreticulin, PDI, and ERp57 are regulated by fluctuation of Ca\(^{2+}\) concentration. For example calreticulin interacts with PDI only when the Ca\(^{2+}\) concentration is below 100 \(\mu\)M, a Ca\(^{2+}\) concentration found upon emptying of the Ca\(^{2+}\) stores (32, 43, 44). The complex dissociates at increased Ca\(^{2+}\) concentration (>500 \(\mu\)M), a concentration common for refilled Ca\(^{2+}\) stores (32, 43, 44). Ca\(^{2+}\) is released from the ER via inositol trisphosphate receptor/Ca\(^{2+}\) channel, and it is taken up by Ca\(^{2+}\)-ATPase (SERCA) (34). In stimulated cells this is a rapid process resulting in continuous changes in the levels of free ER luminal Ca\(^{2+}\) concentrations (32). We show that interaction between calreticulin and PDI and calreticulin and ERp57 are also very rapid with time constants of 0.16 ± 0.01 and 1.4 ± 0.1 s, respectively. Therefore, as far as the kinetics of these protein-protein interactions are concerned, they are physiologically relevant and capable of responding to rapid fluctuations in the luminal Ca\(^{2+}\) concentrations.

Interactions between calreticulin and CB-PDI is restricted to the NH\(_2\)-terminal region (N-domain) of calreticulin, but Ca\(^{2+}\) sensitivity of this interaction is confined to the COOH-terminal, high capacity Ca\(^{2+}\)-binding region (C-domain) of the protein. We also established that ERp57 forms protein complexes with calreticulin, but unlike the calreticulin-PDI complex, binding of calreticulin to ERp57 is initiated by a Ca\(^{2+}\)-independent, conformational change in ERp57 followed by a Ca\(^{2+}\)-
dependent modulation of the complex. Once again the N-domain of calreticulin plays a key role in this protein-protein interaction, and the C-domain is responsible for the Ca\(^{2+}\)-dependent enhancement of the complex. The N-domain of calreticulin is the most conserved region in the protein with over 70% amino acid similarity between human and higher plants (1). One important function of the N-domain of calreticulin may be formation of specific complexes between ER luminal chaperones. The C-domain of the protein may play a Ca\(^{2+}\)-dependent regulatory role in these protein-protein interactions. The C-domain of calreticulin is the least conserved region of the protein, and in some organisms it is even missing (1). Although in all organisms the N-domain of calreticulin may be responsible for formation of protein complexes between different ER chaperones, not all of them will have a Ca\(^{2+}\)-dependent (C-domain-dependent) regulation of these complexes.

What is the physiological relevance of these specific protein-protein interactions? Fig. 9 shows a proposed model for a role of calreticulin and Ca\(^{2+}\) in controlling interactions between ER luminal proteins and regulation of their chaperone function. Under the conditions of empty Ca\(^{2+}\) stores (Fig. 9A), when free Ca\(^{2+}\) concentration is below 100 \(\mu\)M (44), calreticulin will form tight complexes with PDI. This protein-protein interaction may result in inhibition of PDI activity (16). Under low Ca\(^{2+}\) conditions unfolded proteins may be released from PDI to enable them to interact with other chaperones to continue the process of folding and quality control. This may allow a flux of proteins undergoing a folding process, from one chaperone to the other. It is well established that Ca\(^{2+}\) depletion of the ER Ca\(^{2+}\) stores by thapsigargin leads to induction of the unfolded protein pathway (45–50). Results of our work indicate that prolonged Ca\(^{2+}\) depletion of the ER may result in a massive release of unfolded proteins from PDI and calreticulin activating the unfolded protein pathway, without actually changing a total concentration of unfolded proteins.

Under the low Ca\(^{2+}\) conditions calreticulin will also interact with ERp57 (Fig. 9A). These complexes may not be functional, as far as chaperoning is concerned, because at a low Ca\(^{2+}\) concentration calreticulin displays a very weak, if any, binding to monoglucosylated glycoproteins (14). Refilling of Ca\(^{2+}\) stores via function of SERCA will result in significant changes in the dynamics of luminal ER proteins. Under these conditions free Ca\(^{2+}\) concentration will rise above 400 \(\mu\)M (44) followed by a rapid dissociation of calreticulin from PDI (Fig. 9B). Since, under these conditions, PDI does not associate with calreticulin (or calnexin), it is likely that the protein may play an important role in disulfide bond formation of newly synthesized proteins that are not glycosylated. Importantly, elevations in the free Ca\(^{2+}\) concentration in the lumen of the ER will promote calreticulin (and calnexin) lectin-like activity and their interaction with monoglucosylated glycoproteins (Fig. 9B). Calreticulin will recruit ERp57 to provide an “attachment” site for the
Protein to chaperone (disulfide bond formation) newly synthesized glycoproteins. ERp57 chaperone activity is greatly increased when complexed with calreticulin (23). Under these conditions other ER luminal chaperones may also be recruited to further assist in proper folding of newly synthesized glycoproteins. One significant finding is that the high capacity Ca\(^{2+}\) binding site (C-domain) of calreticulin may play a role of a Ca\(^{2+}\) sensor for these protein-protein interactions in response to continuous fluctuations of ER luminal Ca\(^{2+}\) concentrations.

The C-domain of calreticulin is expected to bind Ca\(^{2+}\) only in fully re-filled Ca\(^{2+}\) stores but not under the ER Ca\(^{2+}\) depletion conditions (11). It is likely, therefore, that calreticulin, via its Ca\(^{2+}\) binding ability, may play multiple roles in the lumen of ER: regulation of free Ca\(^{2+}\) concentration and Ca\(^{2+}\)-dependent modulation of chaperone function of other ER luminal proteins such as PDI and ERp57. As previously been reported that the function of other chaperones may be sensitive to fluctuations in the ER luminal Ca\(^{2+}\). At low Ca\(^{2+}\) concentrations in the lumen of the ER, the activity of BiP, another ER luminal chaperone, is also inhibited (51). Protein synthesis,
glycoprotein processing, and transport competence are also blocked under the conditions of ER Ca\textsuperscript{2+} depletion (52–54). Our work suggests that calreticulin may play a key role in the control of these processes.

Calreticulin, from the lumen of the ER, may regulate Ca\textsuperscript{2+} levels in the lumen of ER via potential interactions with the ER Ca\textsuperscript{2+}-ATPase (SERCA) and/or the inositol trisphosphate receptor (5, 10). Favre et al. (55) demonstrated a highly supraregional feedback inhibition of Ca\textsuperscript{2+} uptake via SERCA and postulated existence of an ER lumenal molecule(s) which may regulate SERCA activity (10). Calreticulin may be a potential ER lumenal candidate protein that modulates SERCA activity. Furthermore, calreticulin, from the lumen of the ER, affects steroid-sensitive gene expression (7), cell adhesiveness (6), and store-operated Ca\textsuperscript{2+} influx (29, 30). Results of this work support our earlier hypothesis (13) and suggest that fluctuation of the ER luminal Ca\textsuperscript{2+} concentration will regulate the free concentration of calreticulin in the lumen of the ER. For example, at high luminal Ca\textsuperscript{2+} concentration levels of free calreticulin, in the lumen of the ER, will be significantly increased. Thus, Ca\textsuperscript{2+}-dependent changes in the free calreticulin may play a role in modulation of a variety of calreticulin-dependent signals, including gene expression and Ca\textsuperscript{2+} homeostasis.

In summary, results of this work show that calreticulin appears to be one of the key players of the chaperone network in association with calnexin, PDI, and ERP57. Furthermore, present work indicates that calreticulin may play a role of Ca\textsuperscript{2+} sensor for these chaperones. The protein modulates Ca\textsuperscript{2+}-dependent formation and maintenance of the structural and functional complexes between different ER luminal proteins involved in a variety of steps during protein synthesis, folding, and post-translational modification. It is conceivable that other chaperones may form similar functional complexes with calreticulin and that these interactions may be controlled by hormone-dependent fluctuations of ER luminal free Ca\textsuperscript{2+}.

Acknowledgment—The superb technical assistance of Monika Dabrowska is greatly appreciated.

REFERENCES

1. Michalak, M. (1995) Calreticulin, R. E. Landes Co., Austin, TX
2. Burns, K., Duggan, B., Atkinson, E. A., Famulski, K. S., Nemer, M., Bleackley, R. C., and Michalak, M. (1994) Nature 367, 476–480
3. Dedhar, S., Rennie, P. S., Shago, M., Hagesteijn, C.-Y. L., Filmus, J., Hawley, R. C., and Michalak, M. (1994) Biochim. Biophys. Acta 1209, 310–315
4. Baksh, S., Spamer, C., Oikawa, K., McCanibin, W. E., Heilman, C., Kay, C. M., and Michalak, M. (1995) Biochim. Biophys. Acta 1209, 310–315
5. Bastianutto, C., Clemensi, E., Codazzi, F., Podini, P., Di Giorgi, F., Rizzi, R., Meldolesi, J., and Pozzan, T. (1995) J. Cell Biol. 130, 847–855
6. Mery, L., Mesaeli, N., Michalak, M., Opas, L., Lewi, D. P., and Krause, K. H. (1996) J. Biol. Chem. 271, 9323–9339
7. Lievremont, J.-P., Rizzuto, R., Hendershot, L., and Meldolesi, J. (1997) J. Biol. Chem. 272, 30873–30879
8. Baksh, S., Rizzi, R., Volpe, P., and Meldolesi, J. (1994) Physiol. Rev. 74, 595–636
9. Baksh, S., Bussan, J., and Michalak, M. (1994) FEBS Lett. 357, 57–61
10. Baksh, S., Spamer, C., Oikawa, K., McCanibin, W. E., Heilman, C., Kay, C. M., and Michalak, M. (1995) Biochim. Biophys. Acta 1209, 310–315
11. Baksh, S., Spamer, C., Oikawa, K., McCanibin, W. E., Heilman, C., Kay, C. M., and Michalak, M. (1995) Biochim. Biophys. Acta 1209, 310–315
12. Michalak, M., Milner, R. E., Burns, K., Opas, M., Lewi, D. P., and Krause, K. H. (1996) J. Biol. Chem. 271, 9323–9339
13. Krause, K.-H., and Michalak, M. (1997) Cell 88, 439–443
14. Vassilakos, A., Michalak, M., Lehrman, M. A., and Williams, D. B. (1998) Calreticulin-Protein Interactions