Identification of an N-domain Histidine Essential for Chaperone Function in Calreticulin

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Calreticulin is an endoplasmic reticulum (ER) luminal Ca\(^{2+}\)-binding chaperone involved in folding of newly synthesized glycoproteins via the “calreticulin-calnexin cycle.” We reconstituted ER of calreticulin-deficient cells with N-terminal histidine (His\(^{25}\), His\(^{82}\), His\(^{128}\) and His\(^{153}\)) calreticulin mutants and carried out a functional analysis. In crt\(^{-/-}\) cells bradykinin-dependent Ca\(^{2+}\) release is altered, and the reestablishment of bradykinin-dependent Ca\(^{2+}\) release was used as a marker for calreticulin function. Bradykinin-dependent Ca\(^{2+}\) release from the ER was rescued by wild type calreticulin and by the His\(^{25}\), His\(^{82}\), or His\(^{128}\) mutant but not by the His\(^{153}\) mutant. Wild type calreticulin and the His\(^{25}\), His\(^{82}\), and His\(^{128}\) mutants all prevented in vitro thermal aggregation of malate dehydrogenase and IgY, whereas the His\(^{153}\) mutant did not, indicating that His\(^{153}\) chaperone function was impaired. Biophysical analysis of His\(^{153}\) mutant revealed that conformation changes in calreticulin mutant may be responsible for the loss of its chaperone activity. We conclude that mutation of a single amino acid residue in calreticulin has devastating consequences for its chaperone function, indicating that mutations in chaperones may play a significant role in protein folding disorders.

The endoplasmic reticulum (ER)\(^{1}\) plays an essential role in a variety of cellular processes, including Ca\(^{2+}\) homeostasis, protein and lipid synthesis, and post-translational modification and folding of membrane-associated and secreted proteins (1). The ER ensures that only correctly folded proteins proceed through the secretory pathway and directs misfolded proteins to ER-associated degradation (2, 3). The lumen of the ER is a dynamic environment that contains numerous molecular chaperones and Ca\(^{2+}\)-binding proteins that are designed for these tasks. Molecular chaperones are proteins that bind to misfolded/unfolded proteins in a transient manner to assist in their folding.

Calreticulin is a Ca\(^{2+}\)-binding chaperone that resides in the lumen of the ER and is involved in modulation of Ca\(^{2+}\) homeostasis and in the folding of newly synthesized glycoproteins via the “calreticulin-calnexin cycle” (4–7). Calreticulin and calnexin are both ER lectins, which bind transiently to virtually all newly synthesized glycoproteins (5–7). Chaperone-assisted protein folding has been studied extensively using Escherichia coli GroEL heat shock proteins, which are cytoplasmic (8). Numerous studies have been carried out on ER-associated chaperones (2–7); yet, the molecular features of calreticulin that confer its chaperone function have not yet been determined (7).

Three distinct structural domains have been identified in calreticulin: the amino-terminal, globular N-domain; the central P-domain; and the carboxyl-terminal C-domain (7). NMR (9), modeling (10), and biochemical studies (11) indicate that the globular N-domain and the “extended arm” P-domain of calreticulin may form a functional protein-folding unit (10). This region of calreticulin contains a Zn\(^{2+}\) binding site and one disulfide bond, and it may also bind ATP (12–14). When calreticulin binds Zn\(^{2+}\), it undergoes dramatic conformational changes (15). Chemical modification of calreticulin has revealed that four histidines located in the N-domain of the protein (His\(^{25}\), His\(^{82}\), His\(^{128}\), and His\(^{153}\)) are involved in the Zn\(^{2+}\) binding (12). The Zn\(^{2+}\)-dependent conformational change in calreticulin affects its ability to bind to unfolded protein/glycoprotein substrates in vitro (16), suggesting that conformational changes in calreticulin may modify its chaperone function. The role of the Zn\(^{2+}\) binding histidine residues in calreticulin function is not known.

Calreticulin deficiency is embryonic lethal, and cells derived from calreticulin knockout embryos have impaired Ca\(^{2+}\) homeostasis and compromised protein folding and quality control (11, 17). The availability of calreticulin-deficient cells provides an excellent tool for investigation of the molecular events associated with calreticulin function in the lumen of the ER. In this study, we created site-specific mutants of calreticulin and reconstituted them into the ER lumen of calreticulin-deficient cells. In calreticulin-deficient cells, folding of the bradykinin receptor is altered, which impairs its ability to initiate inositol 1,4,5-trisphosphate-dependent Ca\(^{2+}\) release (11). Therefore, the reestablishment of bradykinin-dependent Ca\(^{2+}\) release from the ER was used as a marker for calreticulin function. Because Zn\(^{2+}\)-dependent conformational changes in calreticulin are critical for its interaction with substrate proteins (16), we focused on the role of histidine residues in calreticulin function. We show that of the histidine residues in the N-domain of calreticulin only His\(^{153}\) is essential for its function.

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¶ The abbreviations used are: ER, endoplasmic reticulum; MDH, malate dehydrogenase; ANS, 8-anilino-1-naphthalene-sulfonic acid; HA, hemagglutinin; MOPS, 4-morpholinepropanesulfonic acid.

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Mutation of a single amino acid residue in calreticulin has devastating consequences for its chaperone function, indicating that mutations in chaperones may play a significant role in protein folding disorders.

**EXPERIMENTAL PROCEDURES**

**Materials**—Trypsin, malate dehydrogenase (MDH), 8-aminol-l-naphthalene-sulfonic acid (ANS), bradykinin, and Dulbecco's modified Eagle's medium were obtained from Sigma. Fetal bovine serum was from Invitrogen. SDS-PAGE reagents and molecular weight makers were from Bio-Rad. Effectene Transfection reagent, Ni$_2^+$-nitrilotriacetic acid-agarose beads was from Qiagen. Zeocin, Pfx DNA polymerase, pBAD/gIII A, and pCDNA3.1/Zeo plasmids were from Invitrogen. EGGEstart IgY purification system was from Promega. All chemicals were of the highest grade available.

**Plasmid and Site-directed Mutagenesis**—For E. coli expression of calreticulin, wild type full-length rabbit calreticulin gene was amplified and cloned into NcoI and XbaI restriction enzyme sites of plasmid pBAD/gIII A (Invitrogen) to generate pBAD-CRT. To express calreticulin in eukaryotic cells, the rabbit calreticulin gene was amplified and cloned into EcoRI and XbaI of pcDNA3.1/Zeo. For easy detection of the recombinant protein, a hemagglutinin epitope (HA) tag was engineered to the C terminus of calreticulin to generate pcDNA-CRT-HA. Site-specific mutagenesis was carried out using a megaprimer polymerase chain reaction technique (18, 19) using Gene Amp PCR system 9700 DNA polymerase. For biophysical and biochemical studies, calreticulin mutant proteins were expressed in E. coli. To generate E. coli expression vector, cDNA encoding calreticulin (Patl-NotI restriction DNA fragment of pcDNA-CRT-HA plasmids) was cloned into Patl-NotI restriction sites of pBAD-CRT plasmid. The following histidine to alanine or histidine deletion mutations were used. Throughout this paper wild type calreticulin and H25A, H82A, H128A, H153A, and H25Del, H82Del, H128Del, and H153Del deletion mutants. Identical results were obtained whether histidine to alanine or histidine deletion mutations were used. Each histidine to alanine or histidine deletion mutant was used. Throughout this paper wild type calreticulin and H25A, H82A, H128A, H153A deletion mutants are designated as CRT-wt and CRT-His$^{25}$, CRT-His$^{82}$, CRT-His$^{128}$, and CRT-His$^{153}$, respectively.

**Cell Culture and Cytoplasmic Ca$^{2+}$ Measurements**—Wild type (K41) and calreticulin-deficient (K42) mouse embryonic fibroblasts were used in this study (11). Cells were grown at 37 °C in a 5% CO$_2$ environment in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. clr$^{-/-}$ cells were transfected with pcDNA3.1/Zeo expression vectors containing cDNA encoding either wild type or mutant calreticulin. Transfections were carried out using Effectene transfection reagent, and stable transfected cell lines were selected with 350 μg/ml Zeocin. The following cell lines expressing wild type calreticulin (clr$^{-/-}$-wt) or specific histidine mutants (clr$^{-/-}$-His$^{25}$, clr$^{-/-}$-His$^{82}$, clr$^{-/-}$-His$^{128}$, and clr$^{-/-}$-His$^{153}$) were generated. For measurement of cytoplasmic Ca$^{2+}$ concentration, 1.5 × 10$^6$ ml were loaded with the fluorescent Ca$^{2+}$ indicator fura-2/AM (2 μM) (11). Ca$^{2+}$ release from internal store was stimulated with 200 nM bradykinin and monitored in a Ca$^{2+}$-free buffer (11).

**Immunofluorescence Microscopy**—Cultured mammalian cells were washed with phosphate-buffered saline and lysed with radioimmune precipitation buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS. Proteins were separated on SDS-PAGE (10% acrylamide) and transferred to nitrocellulose membrane. Western blots were probed with rabbit anti-HA (Roche Applied Science) at 1:300 dilution. Peroxidase-conjugated goat anti rabbit IgG was used as the secondary antibody at 1:10,000 dilution. Cells were grown on 25-mm circular coverslips in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were washed with phosphate-buffered saline and fixed with 4% paraformaldehyde. Goat anti-calreticulin antibodies (1:70 dilution) were used. Texas Red-conjugated donkey anti-goat IgG (1:70 dilution) was used as secondary antibody. Cells were mounted in Vinol 205S and examined with a Bio-Rad confocal fluorescence microscope (model MRC-600) equipped with a krypton/argon laser.

**Expression and Purification of Recombinant Calreticulin**—Proteins were expressed in Top10F E. coli cells in LB medium containing 100 μg of ampicillin/ml. Cultures were grown to the midlog phase followed by the induction of the expression of recombinant proteins with arabinose (final concentration of 0.002%) for 4 h. Cells were spun down at 6,000 rpm for 15 min. The pellet was resuspended in a buffer containing 50 mM Tris, pH 8.0, 300 mM NaCl, 10% glucose and lysed in the French press set at 1,000 p.s.i. followed by centrifugation at 10,000 rpm for 10 min. His-tagged proteins were purified by one-step Ni$^{2+}$-nitrilotriacetic acid-agarose affinity chromatography in native condition. Samples of E. coli lysates were mixed with the Ni$^{2+}$-nitrilotriacetic acid-agarose beads equilibrated with a buffer containing 50 mM Tris, pH 8.0, and 300 mM NaCl, applied onto the column, washed, and eluted with a buffer containing 50 mM Tris, pH 8.0, 300 mM NaCl, and 20 mM imidazole. Recombinant proteins were concentrated by a centrifugal filter (Biomax 30K NMWL membrane; Millipore), and proteins were dissolved in a buffer containing 10 mM Tris, pH 7.0, and 1 mM EDTA. Over 90% of the protein was purified to homogeneity by one-step Ni$^{2+}$-nitrilotriacetic acid-agarose column chromatography. Protein concentration was deter-
determined by a Beckman System 6300 amino acid analyzer or by using Bio-Rad protein assay reagent using bovine serum albumin as a standard (20).

Aggregation Assay—One μM MDH was mixed with varying amounts of wild type and mutant calreticulin at room temperature, and samples were incubated at 45 °C in 50 mM sodium phosphate, pH 7.5 (total volume, 1.2 ml) and monitored for light scattering for 2 h. IgY was isolated from chicken egg yolk according to the protocol of the EGG-strict IgY purification system. IgY was dialyzed overnight against denaturing buffer containing 100 mM Tris, pH 7.0, 6 mM guanidinium hydrochloride, and 40 mM dithiothreitol. The denatured IgY protein (0.25 μM) was suspended in a buffer containing 10 mM Tris-HCl, pH 7.0, 150 mM NaCl, 5 mM CaCl₂, and 40 mM KCl (16) followed by the addition of wild type or mutant calreticulin (0.25 μM). Protein aggregation was induced by increasing sample temperature to 44 °C. Light scattering was measured using a spectrofluorometer system C43/2000 (PTI) equipped with a temperature-controlled cell holder; the excitation and emission wavelengths were set to 320 and 380 nm, respectively.

Intrinsic Fluorescence Measurement and Circular Dichroism—Intrinsic fluorescence measurements were performed at 25 °C in a spectrofluorometer system C43/2000 (Photon Technology International Inc.). Three μM calreticulin wild type and mutant proteins was used for fluorescence measurement in a buffer containing 10 mM MOPS, pH 7.1, 3 mM MgCl₂, and 150 mM KCl (15). The excitation wavelength was set to 286 nm, and the range of emission wavelength was set to 295–450 nm. The effect of Zn²⁺ on the intrinsic fluorescence of protein was evaluated at a wavelength of 334 nm. CD analysis was performed at 25 °C using a Jasco J-720 spectropolarimeter (Jasco Inc., Easton, MD), interfaced to an Epson Equity 386/25 and controlled by Jasco software as described previously (21). The CD spectra were analyzed for secondary structure elements by the Contin ridge regression analysis program of Provencher and Glocner (22).

Proteolytic Digestions—Ten μg of purified, recombinant wild type and mutant calreticulin expressed in E. coli were incubated with trypsin at 1:100 (trypsin/protein; w/w) (14). Aliquots were taken at indicated time points, and the reaction was stopped by the addition of the Laemmli sample buffer (23). The proteins were separated in SDS-PAGE (10% acrylamide) and stained with Coomassie Blue. RESULTS

Expression of Histidine Mutants in Calreticulin-deficient Cells—The N-domain of calreticulin contains 5 histidine residues, which are involved in Zn²⁺ binding to the protein (12). Zn²⁺ binding to calreticulin causes significant conformational changes, which promote the interaction of calreticulin with substrates (12, 14–16, 24, 25). To assess the role of the N-domain histidines in calreticulin function, we mutated residues His²⁵, His⁸², His¹²⁸, and His¹⁵³ to create a series of plasmids containing DNA encoding these mutants and designated as CRT-His²⁵, CRT-His⁸², CRT-His¹²⁸, and CRT-His¹⁵³, respectively. Calreticulin-deficient cells were stably transfected with these expression vectors to create cell lines expressing wild type calreticulin (crt⁻⁻⁻⁻wt) or the specific histidine mutants crt⁻⁻⁻⁻His²⁵, crt⁻⁻⁻⁻His⁸², crt⁻⁻⁻⁻His¹²⁸, and crt⁻⁻⁻⁻His¹⁵³. For easy identification of recombinant proteins, the HA epitope was introduced at the C terminus. We have shown previously that

![](image1.png)

**Fig. 2.** Bradykinin-induced Ca²⁺ release in cells expressing calreticulin mutants. Cells expressing different calreticulin mutants were loaded with the fluorescent Ca²⁺ indicator fura-2 and stimulated with 200 nM bradykinin (11). wt, wild type cells; crt⁻⁻⁻⁻, calreticulin-deficient cells; crt⁻⁻⁻⁻+CRT, calreticulin-deficient cells expressing wild type calreticulin; crt⁻⁻⁻⁻ His²⁵, cells expressing His²⁵ mutant; crt⁻⁻⁻⁻ His⁸², cells expressing His⁸² mutant; crt⁻⁻⁻⁻ His¹²⁸, cells expressing His¹²⁸ mutant; crt⁻⁻⁻⁻ His¹⁵³ cells expressing His¹⁵³ mutant. The amount of Ca²⁺ released by bradykinin is shown. Data are mean ± S.E. (n = 3).

![](image2.png)

**Fig. 3.** Effects of calreticulin mutants on thermal aggregation of MDH, a nonglycosylated substrate. A. SDS-PAGE of recombinant wild type (rCRT) and His¹⁵³ (rCRT-His¹⁵³) mutant of calreticulin used for aggregation studies shown in B. B. MDH was incubated in the presence or absence of wild type calreticulin or calreticulin His⁸² or His¹⁵³ mutant as indicated. Proteins were preincubated at room temperature followed by monitoring aggregation at 45 °C at 360 nm (16). Data are mean ± S.E. (n = 4).
introduction of a C-terminal HA epitope does not affect calreticulin function (11, 26, 27).

Western blot analysis showed that all of the transfected cells expressed recombinant calreticulin (Fig. 1). A relatively lower level of expression of recombinant protein was consistently observed in cells expressing the His25 and His128 mutants (Fig. 1A), and although several different stably transfected cell lines were generated, we were unable to obtain cells with a higher level of expression. Next we carried out immunofluorescence analysis of the cell lines to confirm that the recombinant calreticulin was localized to the ER. All of the cell lines (crt−/−/wt, crt−/−/His25, crt−/−/His82, crt−/−/His128, and crt−/−/His153) expressed recombinant calreticulin, and the protein was localized to an ER-like network (Fig. 1). Morphologically, at a light microscope level, the ER appeared intact in all cell lines as judged by staining with antibodies against PDI, Grp94, and calnexin (data not shown).

**Bradykinin-induced Ca^{2+} Release in Cells Expressing Calreticulin Mutants**—We have shown previously that calreticulin-deficient cells have inhibited bradykinin-dependent Ca^{2+} re-

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**Fig. 4.** Effects of calreticulin on thermal aggregation of IgY, a glycosylated substrate. The effect of calreticulin on the thermal aggregation of IgY was monitored at 44 °C by measuring light scattering at 360 nm as described under "Experimental Procedures" (16). Wild type (wt), His153 (CRT-His153), and His82 (CRT-His82) mutants of calreticulin were tested as indicated in the figure. Data are mean ± S.E. (n = 4).

**Fig. 5.** Conformational changes in His153 mutant. A, intrinsic, tryptophan fluorescence emission spectra analysis of wild type calreticulin (CRT wt) and His153 (CRT His153) mutant was carried out as described under "Experimental Procedures." Intrinsic fluorescence analysis was carried out in the absence and presence of increasing concentrations of Zn^{2+}. B, wild type calreticulin; C, His153 mutant. Kinetics of Zn^{2+}-dependent changes in intrinsic fluorescence of wild type calreticulin (E) or His153 mutant (F). The excitation wavelength was set to 286 nm, and the range of emission wavelength was set to 295–450 nm.
lease, which is restored by expression of full-length recombinant calreticulin (Fig. 2) (11). This is because, in the absence of calreticulin, the bradykinin receptor does not fold properly, so it is unable to bind bradykinin to generate inositol 1,4,5-trisphosphate-dependent signals (11). Consequently, measurement of bradykinin-dependent Ca\(^{2+}\) release from the ER makes an excellent model system to study the function of calreticulin and calreticulin mutants in \(crt\) cells. Therefore, we assessed bradykinin-dependent Ca\(^{2+}\) release in cells expressing the calreticulin mutants we had generated. We performed these experiments with the Ca\(^{2+}\)-sensitive fluorescent dye fura-2. Fig. 2 shows that, as expected, bradykinin caused a rapid and transient increase in the cytoplasmic Ca\(^{2+}\) concentration in wild type cells but not in \(crt\) cells (11). Also, the expression of recombinant calreticulin in \(crt\) cells restored bradykinin-dependent Ca\(^{2+}\) release (Fig. 2, \(crt + CRT\)). Bradykinin-induced Ca\(^{2+}\) release was then measured in the \(crt\) \(-His^{25}, \; ctt\) \(-His^{82}, \; ctt\) \(-His^{128}, \; and \; ctt\) \(-His^{153}\) cell lines. Expression of the \(His^{25}, \; His^{82}, \; and \; His^{128}\) mutants fully restored bradykinin-dependent Ca\(^{2+}\) release (Fig. 2), whereas expression of the \(His^{153}\) mutant did not. This indicates that \(His^{153}\) must play an essential role in calreticulin’s structure and chaperone function.

**His\(^{153}\) Mutant Does Not Prevent Thermal Aggregation of MDH and IgY**—In order to examine the role of \(His^{153}\) in the chaperone activity of calreticulin, we exploited an \(in vitro\) assay used previously by Williams's group (16). This assay makes use of MDH, a nonglycosylated substrate, and IgY, a glycosylated substrate (16). MDH is susceptible to heat-induced aggregation at 45 °C, as measured by light scattering, and it has been widely used as a model substrate in aggregation and refolding assays with many other molecular chaperones (28–30). Williams's group has shown recently that full-length recombinant calreticulin efficiently prevents MDH or IgY thermal-induced aggregation \(in vitro\) (16). To further investigate the role of histidine residues in calreticulin function, we examined the effectiveness of the \(His^{25}, \; His^{82}, \; His^{128}, \; and \; His^{153}\) mutants in prevention of thermal aggregation of MDH and IgY, \(in vitro\). In order to do this, we expressed the recombinant proteins in \(E. \; coli\) and purified them.

Fig. 3 shows that one-step purification of the recombinant proteins on a nickel column was sufficient. As previously reported, when MDH was heated to 45 °C it began to form insoluble aggregates that could be detected by light scattering (Fig. 3) (16, 28–30). As expected, aggregation was reduced in the presence of different concentrations of wild type calreticulin...
Site-specific Mutation of Calreticulin

(16). However, the addition of the His^{153} calreticulin mutant to 0.1 μM (calreticulin/MDH = 0.1:1) or 0.2 μM (calreticulin/MDH = 0.2:1) did not prevent MDH aggregation (Fig. 3B). In contrast, His^{82} mutant prevented aggregation of MDH similarly to wild type protein (Fig. 3B). In control experiments, the His^{82} and His^{128} mutants were also expressed and purified, and their activity was tested in the MDH assay. As we expected, these mutants all prevented thermal aggregation of MDH similarly to wild type calreticulin (data not shown).

Next we tested the effectiveness of calreticulin mutants in preventing aggregation of a glycosylated substrate, IgY. The majority of the effects of calreticulin on IgY aggregation are mediated by calreticulin-carbohydrate interactions (16). As expected, full-length calreticulin effectively prevented aggregation of chemically denatured IgY (Fig. 4). The His^{25}, His^{82} and His^{128} mutants were also effective in preventing aggregation of IgY (not shown). However, in keeping with the results of the MDH-refolding experiments (Fig. 3), the His^{153} mutant did not prevent aggregation of IgY (Fig. 4).

Structural Analysis of Calreticulin Mutants—Molecular modeling of the structure of calreticulin, based on the known crystal structure of calnexin (31), shows that His^{153} is located in a “pocket” close to a putative carbohydrate/substrate binding site (see Fig. 9). This region is involved in Zn^{2+} binding to calreticulin (12), so mutation of this His^{153} residue may cause Zn^{2+}-dependent conformational changes in the protein, affecting its interactions with its substrates (16). To test this hypothesis, we carried out fluorescence and CD analysis and limited proteolysis of wild type and mutant calreticulins. First we measured the intrinsic fluorescence of recombinant calreticulin and the calreticulin mutants. The intrinsic fluorescence emission of a protein is affected by the movement of charged groups and by hydrophobic changes in the microenvironment, particularly those resulting from movement of tryptophan residues into the solvent. Thus, the emission scan provides information on changes in tertiary structures. If the protein is excited at 286 nm, where absorption is predominantly by tryptophan, the emission maximum at 334 nm represents the contribution of tryptophan.

Fluorescence emission spectra were measured for wild type calreticulin and for calreticulin mutants at varying Zn^{2+} concentrations (Fig. 5). Fig. 5A shows that in the absence of Zn^{2+} both wild type calreticulin and the His^{153} mutant have an emission maximum wavelength of 334 nm. However, the basal intrinsic fluorescence intensity (λ_{max}) of wild type calreticulin was over 30% higher than that observed for the His^{153} mutant (Fig. 5). This indicates that the mutant protein has less tryptophan exposed to the solvent. In the presence of increasing concentrations of Zn^{2+}, there was a significant increase in the intensity of fluorescence from wild type calreticulin (Fig. 5, B and D), indicating Zn^{2+}-dependent conformational changes in the protein. In contrast, the His^{153} calreticulin mutant showed only small changes in intrinsic fluorescence as a function of increasing Zn^{2+} concentration (Fig. 5, C and D). This indicates that the effect of Zn^{2+} on calreticulin structure and conformation is compromised in the absence of His^{153}. Ca^{2+}-dependent changes in intrinsic fluorescence were also measured and showed no significant difference between wild type calreticulin and calreticulin His^{153} mutant (Fig. 5, E and F).

ANS has been used extensively to monitor hydrophobic sites on the surface of proteins. When ANS is exposed to a hydrophobic environment, its fluorescent intensity is enhanced, and
significant effect on the structure of calreticulin. Both proteins His153 mutant calreticulin resulted in reduced emission spectrum from 498 to 473 nm (Fig. 6), and the addition of Zn$^{2+}$ induced a further slight blue shift from 473 to 468 nm (Fig. 6). The enhancement of ANS fluorescent intensity was dose-dependent (data not shown). At 468 nm, the addition of wild type calreticulin induced a 4.5-fold increase in ANS fluorescent intensity was compared with only 4.5-fold for His153 mutant (Fig. 6). This indicates that the His153 calreticulin mutant has a less hydrophobic surface than wild type calreticulin.

To determine whether the observed differences in fluorescence behavior (Figs. 5 and 6) reflect conformational differences between the wild type and His153 mutant calreticulins, we carried out CD analysis of the purified proteins. Fig. 7 shows that the CD spectra of calreticulin and the His153 mutant are very similar in shape. The Contin program for calculating secondary structural elements (Table I) estimates the α-helical content for both proteins as 12–14%. In contrast, the combined β-sheet and β-turn content for the two proteins differs significantly, at 58% for wild type calreticulin and 73% for the His153 mutant. This general increase in β-sheet-β-turn in the mutant protein supports the findings of our fluorescence experiments and indicates that mutation of His153 has a significant effect on the structure of calreticulin. Both proteins underwent a conformational change upon the addition of Zn$^{2+}$, with a reduction in the amount of α-helix (Table I, Fig. 7). The addition of Zn$^{2+}$ to wild type calreticulin resulted in fewer β-sheet structures and an increase in β-turn and unordered structures (Table I). In contrast, the addition of Zn$^{2+}$ to the His153 mutant calreticulin resulted in reduced β-sheet-β-turn content and a significant increase in unordered structures (Table I).

Finally, we investigated conformational differences between calreticulin and calreticulin mutants using limited proteolysis. We have shown previously that the resistance of recombinant calreticulin to trypsin is an excellent measure of its folding (14). Here we examined the effect of His153 mutation on calreticulin’s trypsin resistance. Purified proteins were treated with trypsin (trypsin/calreticulin = 1:100) for up to 20 min and then analyzed by SDS-PAGE. Fig. 8 shows that a significant proportion of wild type calreticulin was resistant to trypsin digestion for up to 10 min. In contrast, the His153 calreticulin mutant was completely degraded and undetectable after 5 min of treatment with the trypsin. This increased susceptibility to proteolysis again indicates that mutation of His153 in calreticulin leads to significant alteration of its structure.

**DISCUSSION**

It is well established that the ER plays a critical role in the folding of secretory and membrane-associated proteins and glycoproteins. The ER contains several unique groups of molecular chaperones, including disulfide isomerases, immunophilins, the Hsp70 and Hsp90 family proteins, and the lectins calreticulin and calnexin (32). To date, it has been difficult to investigate the function of these chaperones at a structural level, but the recent availability of calreticulin-deficient cells has provided us with a unique tool to investigate the molecular features of calreticulin that are required for its chaperone function. In this study, we reconstituted the ER of calreticulin-deficient cells with calreticulin mutants and then performed a functional analysis in vitro. This was followed by *in vitro* investigation of the structure of various purified, recombinant calreticulin mutants to assess the effect of specific mutations on the protein structure and function.

We focused on the N-terminal histidine residues because of their known role in Zn$^{2+}$ binding to calreticulin and, most importantly, because Zn$^{2+}$-dependent conformational changes are critical for calreticulin-substrate interactions (16). Remarkably, mutation of just one histidine residue (His153) abolished calreticulin function as measured by its ability to rescue the crt$^{-/-}$ phenotype. Calreticulin-deficient cells have impaired bradykinin-dependent Ca$^{2+}$ release (11). In these cells, bradykinin does not bind to the cell surface bradykinin receptor, probably because the receptor is incorrectly folded (11). Bradykinin-dependent Ca$^{2+}$ release from the ER was rescued by transfection with wild type calreticulin (11) and calreticulin with mutated His25, His82, or His128. In contrast, bradykinin-induced Ca$^{2+}$ release in crt$^{-/-}$ cells was not rescued by transfection with the His153 mutant. Studies on the activity of purified recombinant calreticulin and the calreticulin mutants showed that wild type calreticulin and the His153, His82, and His128 mutants all prevented *in vitro* thermal aggregation of MDH and IgY, whereas the His153 mutant did not. We conclude that His153 is essential for the chaperone function of calreticulin.

To investigate the molecular mechanism behind the loss of

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**TABLE I**

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<td>0.23</td>
<td>0.15</td>
<td>0.999</td>
</tr>
<tr>
<td>CRT His153</td>
<td>0.09</td>
<td>0.46</td>
<td>0.19</td>
<td>0.27</td>
<td>0.999</td>
</tr>
</tbody>
</table>

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![Fig. 8. Trypsin digestion of calreticulin and His153 mutant.](http://www.jbc.org/)

_A. Trypsin digestion of calreticulin (A) and His153 calreticulin mutant (B) were expressed in E. coli, and the purified proteins were incubated with trypsin at 1:100 (trypsin/protein; w/w) at 37 °C. Aliquots were taken at the time points indicated, and the proteins were separated by SDS-PAGE and stained with Coomassie Blue._

_B. Aliquots were taken at the time points indicated, and the proteins were separated by SDS-PAGE and stained with Coomassie Blue._

_N.B._ MDH and IgY, whereas the His153 mutant did not. We conclude that His153 is essential for the chaperone function of calreticulin.
chaperone activity in the His153 mutant, we used intrinsic fluorescence measurements, CD analysis, and limited proteolysis. These studies enabled us to determine whether the His153 mutation affects the biophysical properties of calreticulin. The reduced intrinsic fluorescence of the His153 mutant indicates that some local conformational changes may have occurred in the mutant calreticulin. Importantly, Zn$^{2+}$-dependent conformational changes, a signature of calreticulin behavior (15), were also compromised in the His153 mutant. This agrees with our previous observation that the N-domain histidine residues are involved in Zn$^{2+}$ binding to calreticulin (12). The ANS fluorescence analysis indicated that the His153 mutant has a significantly lower surface hydrophobicity than wild type calreticulin. Finally, our CD analysis revealed that the His153 mutant differs significantly from wild type calreticulin in $\beta$-sheet $\beta$-turn content, and the limited proteolysis studies indicated that the His153 mutant is more susceptible to trypsin digestion that the wild type protein. Clearly, the His153 mutation results in local changes in calreticulin conformation, and these severely affect its ability to function as a molecular chaperone.

We have modeled the three-dimensional structure of calreticulin (10) based on crystallographic data available for calnexin (31) and NMR data available for the P-domain of calreticulin (33). This model provides an excellent framework for interpretation of our results and for determining the role of the His153-containing region (loop) in protein folding (Fig. 9). The crystal structure of calnexin reveals that it consists of a globular $\beta$-sandwich domain and an elongated arm P-domain forming an extended arm containing the repeat motifs (31). The central, P-domain of calreticulin also forms an elongated arm-like structure (9). The extended arm is curved, forming an opening that probably forms a substrate binding site, including the carbohydrate-binding site (31). Our three-dimensional model of calreticulin’s N-terminal globular domain and proline-rich, central P-domain (Fig. 9) shows that the N-terminal domain is predicted to form a globular $\beta$-sheet structure (10). Together with the extended arm P-domain, they form a folding unit in calreticulin (10, 11).

Importantly, our model of calreticulin’s structure has helped us to visualize the location of the N-terminal histidine residues investigated in this study (Fig. 9). His273, His82, His106, and His128 are all found on the outer surface of the globular N-domain of the protein, away from the extended arm structure of the P-domain (Fig. 9). These residues appear to be located away from the substrate binding region in calreticulin; therefore, their mutation or deletion has no effect on calreticulin’s function as a molecular chaperone. In contrast, His153 is localized differently within calreticulin (Fig. 9). It is found on the top of a loop that is part of a short $\beta$-strand located at the interface between the globular N-domain and the “extended arm” P-domain (Fig. 9). Importantly, the loop containing His153 is predicted to be flexible with some lateral mobility toward the extend arm of the P-domain. This flexibility may significantly influence the shape of the substrate (carbohydrate) binding pocket (Fig. 9).

Calreticulin and calnexin are lectin-like chaperones with a similar specificity for monoglucosylated carbohydrate. However, there are a number of protein substrates that are unique to either calreticulin or calnexin (7), and it is not obvious what determines this substrate specificity. It is possible that the histidine residues in the N-domains of the two proteins play a role, since there are 9 histidine residues in the N-terminal globular domain of calnexin (31, 34), and only 2 of these are conserved in calreticulin. The conserved residues are His128, which had no effect on calreticulin function in this study, and His153 (His273 in calnexin). Although the His153 is conserved in calnexin, the amino acid sequences that flank this critical residue differ in the two proteins. For example, upstream of His273 in calnexin, there are 10 amino acid residues that form a short $\alpha$-helix not found in calreticulin (31). The loop containing His273/His153 is, therefore, significantly longer in calnexin...
Site-specific Mutation of Calreticulin

than in calreticulin and may have different mobility. It is thus conceivable that the “loop” that contains the His153 residue may contribute to determination of calreticulin’s substrate specificity.

In summary, our work indicates that a single amino acid mutation in calreticulin, an ER luminal chaperone, significantly affects protein folding. It follows that a mutation such as this could result in a genetic disorder of protein folding. This finding has important implications in our understanding of protein folding diseases.

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Identification of an N-domain Histidine Essential for Chaperone Function in Calreticulin
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