Cartilage Oligomeric Matrix Protein Is a Calcium-binding Protein, and a Mutation in Its Type 3 Repeats Causes Conformational Changes

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Mutations in residues in the type 3 calcium-binding repeats and COOH-terminal globular region of cartilage oligomeric matrix protein (COMP) lead to two skeletal dysplasias, pseudoachondroplasia and multiple epiphyseal dysplasia. It has been hypothesized that these mutations cause COMP to misfold and to be retained in the endoplasmic reticulum. However, this hypothesis is not supported by previous reports that COMP, when purified in the presence of EDTA, shows no obvious difference in electron microscopic appearance in the presence or absence of calcium ions. Since this discrepancy may be due to the removal of calcium during purification, we have expressed wild-type COMP and the most common mutant form found in pseudoachondroplasia, MUT3, using a mammalian expression system and have purified both proteins in the presence of calcium. Both proteins are expressed as pentamers. Direct calcium binding experiments demonstrate that wild-type COMP, when purified in the presence of calcium, is a calcium-binding protein. Rotary shadowing electron microscopy and limited trypsin digestion at various calcium concentrations show that there are conformational changes associated with calcium binding to COMP. Whereas COMP exists in a more compact conformation in the presence of calcium, it shows a more extended conformation when calcium is removed. MUT3, with a single aspartic acid deletion in the type 3 repeats, binds less calcium and presents an intermediate conformation between the calcium-replete and calcium-depleted forms of COMP. In conclusion, we show that a single mutation in the type 3 repeats of COMP causes the mutant protein to misfold. Our data demonstrate the importance of calcium binding to the structure of COMP and provide a plausible explanation for the observation that mutations in the type 3 repeats and COOH-terminal globular region lead to pseudoachondroplasia.

Cartilage oligomeric matrix protein (COMP) is a pentameric extracellular matrix protein with a subunit size of 95–97 kDa (Ref. 1 and this study). It is primarily localized in the chondrocyte extracellular matrix and can also be found in synovium, tendon, and ligament (2–5). COMP is the fifth member of the thrombospondin (TSP) family (6, 7). It has a coiled-coil region responsible for multimerization and interchain disulfide bonds, four epidermal growth factor-like type 2 repeats, seven highly conserved type 3 repeats that consist of 13 calcium-binding loops, and a COOH-terminal globular domain (6, 7).

Although the function of COMP is uncertain, its importance is suggested by findings that COMP mutations lead to human skeletal dysplasias, including pseudoachondroplasia (PSACH) and multiple epiphyseal dysplasia Fairbank and Ribbing types (EDM1). Whereas most of the mutations are located in the type 3 calcium-binding repeats, some are found in the C-globe region (8–14). PSACH and EDM1 are autosomal dominant skeletal dysplasias. Clinically, PSACH patients show disproportionate short stature, joint laxity, and early onset osteoarthritis (15, 16). EDM1 patients show mild short stature and joint pain, particularly in the hips, in addition to early onset osteoarthritis (16, 17). In ultrastructural studies, chondrocytes from PSACH and EDM1 patients are characterized by enormous vesicles formed from the rough endoplasmic reticulum (ER) that have a unique lamellar appearance with alternating electron-lucent and electron-dense layers (15, 17–20). Extracellular matrix components, including the major cartilage proteoglycan aggrecan, have been reported to be retained in these vesicles (17). In PSACH and EDM1 patients with COMP mutations, type IX collagen and COMP itself have been shown to be trapped in these enlarged vesicles (20, 21). The retention appeared to be cell type-specific since COMP is secreted normally from patient tendon and ligament in vivo and patient chondrocytes cultured in monolayer (20–22). It is unclear how these mutations affect COMP and why mutations in COMP lead to the intracellular retention of various proteins and the disease phenotype.

The large number of consecutive calcium-binding consensus repeats is unique to TSPs. The aspartate-rich sequences are similar to sequences in calcium-binding sites of a class of calcium-binding proteins including calmodulin, parvalbumin, and fibrinogen (23). Variations of the calcium-binding consensus sequence DX(D/E)GXX(D/E)DXXDX occur 13 times in a TSP subunit and have been proposed to form calcium-binding loops (23–25). Equilibrium dialysis, circular dichroism, and limited trypsin digestion studies on TSP1 suggest that each TSP1 subunit binds 11–12 calcium ions in a cooperative fashion. Most of the protein; TSP, thrombospondin; PSACH, pseudoachondroplasia; EDM1, multiple epiphyseal dysplasia caused by COMP mutations; ER, endoplasmic reticulum; TBS, Tris-buffered saline; PAGE, polyacrylamide gel electrophoresis; EM, electron microscopy/microscopic.
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Calcium-binding activity can be attributed to the type 3 repeats. These studies also suggest that there is a conformational change when TSP1 binds calcium (25, 26). The type 3 repeats are highly conserved in all TSP family members. According to the presence of the type 3 repeats in COMP (see Fig. 1) and studies on TSP1, COMP is predicted to bind calcium and to display different conformations in the presence and absence of calcium. However, in all previous reports, COMP, originally purified in the presence of EDTA, failed to show a significant conformational difference as a function of calcium (27–30), therefore casting doubt on whether COMP itself is a calcium-binding protein.

It has been hypothesized that mutations in the type 3 repeats of COMP result in the incorrect folding of the protein, which leads to the retention of the protein in the ER. However, previous results question why mutations in the calcium-binding repeats should lead to such significant disease phenotypes if the presence and absence of calcium in the molecule are not associated with any conformational differences. Therefore, in this study, we set out to reexamine the effect of calcium on the structure and function of COMP. To preserve possible calcium-sensitive structures that might be perturbed by treatment with EDTA as in previous purification methods, we chose not to study COMP or MUT3 purified from natural sources. Instead, we have expressed human COMP and MUT3 and purified them from conditioned media in the presence of calcium. We show that COMP, when purified in the presence of calcium, is a calcium-binding protein. COMP also displays different conformations as a function of calcium concentration, demonstrating that the most common mutation found in PSACH, MUT3, a single amino acid deletion, is sufficient to cause the mutant protein to bind less calcium and to perturb the structure. These results are contrary to all previously published studies, which have not detected any effect of calcium on COMP conformation using COMP purified in the presence of EDTA (27–30). Our results argue for the importance of the type 3 calcium-binding repeats of COMP and serve as a basis for comprehending the mechanisms of mutations in this region leading to PSACH and EDM1.

MATERIALS AND METHODS

Cloning of MUT3—The typical PSACH mutation, MUT3, was previously identified in patient P303 from family P300F (9). MUT3 has a 3-base pair GAC deletion in nucleotides 1430–1444, resulting in a single aspartate deletion in the five aspartate residues in the tenth and eleventh calcium-binding loops (Fig. 1). It is the most common mutation, accounting for 30% of the PSACH cases (9, 11). Total RNA was isolated from patient lymphocytes, and cDNA was transcribed using the Superscript preamplification system (Life Technologies, Inc.). The cDNA sample was amplified following standard procedures using primers L6F (5′-TGGAGACGGACATCAGGACT-3′) and L4R (5′-CTCGGC-CACAGCAGGAAGG-3′). The 580-base pair product was cloned into the pGEM-T vector (Promega, Madison, WI). Clones were screened for the deletion by polymerase chain reaction amplification with primers L6F and L6R (9). Clone 5 contains the deletion.

The full-length COMP cDNA was cloned previously and sequenced (7). The Bul/Ste1 fragment of COMP was replaced with the mutant fragment from clone 5. The resulting clone, MUT3, was confirmed by sequence analysis with primers L6F and L4R using an Applied Biosystems automated sequencer.

COMP and MUT3 Protein Purification—Full-length COMP and MUT3 sequences were cloned into pcDNA3.1+ vectors (Invitrogen, Carlsbad, CA). The resulting respective clones were transfected into human embryonic kidney cells (293 cells) using Lipofectin reagent (Life Technologies, Inc.). Single colonies of stable transfectants were isolated using selection with G418 and expansion. Cells were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) plus 10% heat-inactivated fetal bovine serum until nearly confluent. Cells were then washed twice with Dulbecco’s modified Eagle’s medium and grown in Dulbecco’s modified Eagle’s medium plus 2 mM l-glutamine for 48 h. Conditioned media were collected for purification. To purify COMP or MUT3, conditioned media were precipitated by 30% ammonium sulfate at 4 °C overnight. Precipitates were resuspended in Tris-buffered saline (TBS) (pH 7.5) containing 2 mM CaCl2, loaded onto linear 10–20% sucrose gradients in TBS with 2 mM CaCl2, and separated by centrifugation in a Beckman SW41 Ti rotor at 58,000 rpm for 20–24 h. Fractions containing COMP or MUT3 were identified by SDS-polyacrylamide gel electrophoresis (PAGE) analysis and pooled. Fractions were stored at −80 °C until further use.

Electron Microscopy of Human COMP and MUT3—Purified COMP or MUT3 in buffer containing 2 mM CaCl2 was diluted 1:5 with 70% glycerol and 0.15 M ammonium acetate containing 0.2 or 1 mM CaCl2 prior to spraying. The final calcium concentration in the samples was therefore either 0.56 or 1.2 mM. An equivalent sample of protein was adjusted to 5 mM EDTA prior to mixing with 70% glycerol and 0.15 M ammonium acetate prepared without CaCl2. The samples were sprayed onto freshly cleaved mica and rotary-shadowed with platinum from an angle of 6° to the horizontal. A shadow thickness of ~12 Å was used, and the samples were coated with 100-A thick carbon film from an angle of 90°. Replicas of COMP and MUT3 were observed on a transmission electron microscope, and their pictures were taken for further examination.

Limited Trypsin Digestion of COMP and MUT3 at Varying Calcium Concentrations—Purified recombinant COMP or MUT3 was dialyzed against TBS containing either 2 or 0.5 mM CaCl2. EDTA was added to samples of each protein to achieve the desired final free Ca2+ concentration. Limited trypsin digestion was carried out in a ratio of 1:100 for 20 h at 0 °C. Digestion was stopped by adding SDS sample buffer to the samples, followed by boiling of the samples and SDS-PAGE. Digestion was also performed in the presence of 2 mM N-ethylmaleimide. To sequence limited trypptic fragments, proteins with specific free Ca2+ concentrations were digested with trypsin, separated by SDS-PAGE, and electrophotographically transferred to a piece of polivinylidene difluoride membrane (Bio-Rad). Protein bands were visualized by brief Ponceau S staining and cut out. After extensive washing with deionized distilled water, the polivinylidene difluoride membrane strips with the tryptic bands were sent to the Harvard Microchemistry Facility (Cambridge, MA) for amino acid analysis and NH2-terminal sequencing.

Calcium Binding to COMP and MUT3—Calcium binding to COMP and MUT3 at 0.3 and 1.2 mM free calcium was measured. For binding studies, COMP and MUT3 were purified as described above, except that all solutions contained 0.3 instead of 2 mM CaCl2. All glassware was soaked in deionized distilled water before use. COMP and MUT3 (100 µl each), purified in the presence of either 0.3 or 2 mM CaCl2, along with 100 µl of TSP1, were dialyzed in Slide-A-Lyzer mini-dialysis units (Pierce) against 9.7 ml of dialysis buffer for 24 h at 4 °C. The dialysis buffer contained 140 mM NaCl, 17% sucrose, 0.3 or 1.2 mM CaCl2, and 10 µCi/ml 45CaCl2 (NEL Life Science Products). At the end of dialysis, four 10-µl samples of each protein and dialysis buffer were taken for scintillation counting, and four-10-µl samples for protein determination using Bio-Rad protein assays. COMP, reduced by 5 mM dithiothreitol, was sent to the Harvard Microchemistry Facility for matrix-assisted laser desorption time-of-flight mass spectrometry analysis. An average molecular weight of 84.8 × 103/monomeric subunit was obtained and was used for calcium-binding calculation with COMP and MUT3. A molecular weight of 150 × 103/subunit was used for the TSP1 calculation.
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RESULTS

When human embryonic kidney 293 cells were transfected with full-length COMP or MUT3 DNA, they were able to process and secrete both proteins. The purification procedure produced 2.8 ± 2.1 μg of purified COMP and 1.5 ± 0.9 μg of purified MUT3 per ml of serum-free conditioned medium (mean ± S.D. from >10 purification procedures). Untransformed 293 cells did not synthesize any endogenous COMP as judged by immunoblotting with a polyclonal antibody (F8) against COMP (22) (data not shown). The two purified proteins migrated with equivalent molecular sizes when analyzed by SDS-PAGE under both nonreducing and reducing conditions. FN, fibronectin.

Although COMP and MUT3 appeared to have the same molecular weight in SDS-PAGE, differences became apparent during purification. When COMP and MUT3 were subjected to sedimentation through linear 10–20% sucrose gradients in the presence of 2 mM CaCl2, the peak of COMP appeared in earlier fractions from the gradient than that of MUT3 (Fig. 3). This suggests that although COMP and MUT3 have almost the same molecular mass, their structures, as reflected in their sedimentation, are different.

The rotary shadowing EM images, in which both COMP and MUT3 were visualized as pentamers (Fig. 4), suggested that mutations in the type 3 repeat region did not affect the subunit assembly. This conclusion was consistent with the observation that COMP and MUT3 comigrated in the absence of reducing agent in SDS-PAGE (Fig. 2). In the presence of calcium, COMP had a compact appearance that made it difficult to resolve the five subunits. In the presence of EDTA, COMP adopted an extended conformation (Fig. 4). The uncorrected lengths of the arms of COMP from the interchain disulfides to the end of the C-globe were 30.4 ± 3.3 nm in the presence of EDTA, 19.9 ± 2.6 nm in the presence of 0.56 mM calcium, and 19.3 ± 4.8 nm in the presence of 1.2 mM calcium (mean ± S.D. of 20 measurements each). These results suggested that COMP, when purified in a calcium-replete form, will undergo conformational changes when calcium is chelated from the molecules. This observation differs from previously published reports on COMP purified in the presence of EDTA (28, 29). However, the conformational change we observed was consistent with previous findings on TSP1, TSP3, and TSP4 and with the presence of the highly homologous type 3 calcium-binding repeats in COMP (6, 7, 31–33). Under EM, MUT3 appeared indistinguishable from COMP in the presence of EDTA (Fig. 4), with an arm length of 31.9 ± 4.4 nm. However, in the presence of calcium, MUT3 adopted an intermediate conformation between the calcium-replete and calcium-depleted forms of COMP, with arm lengths of 25.6 ± 3.8 and 25.5 ± 3.9 nm in the presence of 0.56 and 1.2 mM calcium, respectively. MUT3 was not as tightly folded as COMP in the calcium-replete form, whereas it was more compact than the calcium-depleted form (Fig. 4).

To further probe the conformational differences between COMP and MUT3, we employed limited trypsin digestion of COMP and MUT3 under various calcium concentrations (Fig. 5). Five tryptic fragments with molecular masses of 27, 36, 50, 55, and 63 kDa under reducing conditions were observed in the digestion of both COMP and MUT3. Trypsin digestion of COMP generated one additional fragment of 67 kDa that was not present in the MUT3 digestion at the enzyme/substrate ratio employed. At low calcium concentrations (in the range of 1–25 μM), both COMP and MUT3 were readily cleaved by trypsin into two small fragments of 27 and 36 kDa (Fig. 5). When the calcium concentration was increased to the range of 50–100 μM, COMP was digested into intermediate bands of 50 and 55 kDa. When the calcium concentration was higher than 150 μM,
with increasing calcium concentration, the 63- and 67-kDa bands of COMP started to appear. The 67-kDa band became the prevalent band at calcium concentrations in the millimolar range. With MUT3, the intermediate bands of 50 and 55 kDa started to appear at 50 \(\text{mM} \text{Ca}^{2+}\). However, these bands persisted in a much wider range of calcium concentrations (up to 500 \(\text{mM}\)) compared with COMP. The calcium concentrations given were not corrected for calcium bound to COMP or MUT3. Shown in A are the samples in a final calcium concentration of 0.56 mM. Similar images were observed with a final calcium concentration of 1.2 mM. Shown in B are equivalent samples of protein adjusted to 5 mM EDTA prior to mixing with 70% glycerol and 0.15 mM ammonium acetate prepared without CaCl\(_2\). The samples were sprayed onto freshly cleaved mica and rotary-shadowed with platinum as described under “Materials and Methods.” The bars equal 50 nm.

**Fig. 4.** Electron microscopy of recombinant human COMP and MUT3. Purified COMP (C) and MUT3 (M) proteins in Tris-buffered saline containing 2 mM CaCl\(_2\), were diluted 1:5 with 70% glycerol, 0.15 mM ammonium acetate, and 0.2 or 1 mM CaCl\(_2\) prior to spraying. The final calcium concentration in the samples was either 0.56 or 1.2 mM (the calcium concentrations given were not corrected for calcium bound to COMP or MUT3). Shown in A are the samples in a final calcium concentration of 0.56 mM. Similar images were observed with a final calcium concentration of 1.2 mM. Shown in B are equivalent samples of protein adjusted to 5 mM EDTA prior to mixing with 70% glycerol and 0.15 mM ammonium acetate prepared without CaCl\(_2\). The samples were sprayed onto freshly cleaved mica and rotary-shadowed with platinum as described under “Materials and Methods.” The bars equal 50 nm.

**Fig. 5.** Limited trypsin digestion of COMP and MUT3 at varying calcium concentrations. Purified recombinant COMP or MUT3 was dialyzed against Tris-buffered saline containing either 2 or 0.5 mM CaCl\(_2\). EDTA was added to 16 \(\mu\)g of each protein so that the final free \(\text{Ca}^{2+}\) concentrations were as indicated in millimolar. The calcium concentrations given were not corrected for calcium bound to COMP or MUT3. Trypsin digestion was carried out at an enzyme/substrate ratio of 1:100 for 20 h at 0 °C. Digestion was stopped by adding reducing SDS sample buffer, and the polypeptides were separated by SDS-PAGE. Molecular mass markers are as indicated in kilodaltons. ND, non-digested proteins.

Electron microscopy of recombinant human COMP and MUT3. Purified COMP (C) and MUT3 (M) proteins in Tris-buffered saline containing 2 mM CaCl\(_2\), were diluted 1:5 with 70% glycerol, 0.15 mM ammonium acetate, and 0.2 or 1 mM CaCl\(_2\) prior to spraying. The final calcium concentration in the samples was either 0.56 or 1.2 mM (the calcium concentrations given were not corrected for calcium bound to COMP or MUT3). Shown in A are the samples in a final calcium concentration of 0.56 mM. Similar images were observed with a final calcium concentration of 1.2 mM. Shown in B are equivalent samples of protein adjusted to 5 mM EDTA prior to mixing with 70% glycerol and 0.15 mM ammonium acetate prepared without CaCl\(_2\). The samples were sprayed onto freshly cleaved mica and rotary-shadowed with platinum as described under “Materials and Methods.” The bars equal 50 nm.

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We performed amino acid composition and NH₂-terminal amino acid sequencing analyses on limited trypsin digests of COMP and MUT3 to determine the sites of digestion in these two molecules. The 67-, 63-, 50-, and 27-kDa bands of COMP and the 63-, 50-, and 27-kDa bands of MUT3 were submitted for sequencing. All bands except for the 27-kDa band of MUT3 had the NH₂-terminal amino acid sequence of TGLPSVRPLL. This showed that trypsin cleaved between Arg 79 and Thr 80 after the interchain disulfide bonds and before the start of the epidermal growth factor-like type 2 repeats. Thus, after initial trypsin cleavage of COMP, further digestion proceeded from the carboxyl terminus in a calcium-dependent fashion, with MUT3 more susceptible to digestion in calcium than COMP. The 27-kDa band of MUT3 had essentially the same NH₂-terminal amino acid sequence and lacked only the NH₂-terminal threonine residue (GLPSVRPLL).

To establish that the conformational change that is observed by electron microscopy, sucrose gradient centrifugation, and limited trypsin digest reflected decreased calcium binding in MUT3, equilibrium dialysis was used to directly assess calcium binding to COMP and MUT3. At 0.3 and 1.2 mM free calcium, each TSP1 subunit bound 10⁻⁶ and 10⁻⁵ calcium ions, respectively (mean ± S.D.). This is in good agreement with previous reports where 11–12 calcium ions were reported to bind to each TSP1 subunit (25, 26). At 0.3 mM free calcium, each subunit of COMP bound 11 ± 1 calcium ions, whereas each MUT3 subunit bound 7 ± 1 calcium ions (p < 0.0001). At 1.2 mM free calcium, each COMP subunit bound 9 ± 1 calcium ions, and each MUT3 subunit bound 7 ± 1 calcium ions (p < 0.0005) (Fig. 7). These data suggest that a single amino acid mutation in the type 3 calcium-binding domain of COMP reduces its overall calcium-binding capacity.

DISCUSSION

In this study, we have explored the mechanisms by which mutations in COMP lead to PSACH and EDM1. For this purpose, we chose to compare the structural differences between COMP and its most common mutation in PSACH, MUT3. Both recombinant proteins are processed and secreted into the culture supernatant as pentamers by 293 cells. This suggests that the mutation that is located in the type 3 calcium-binding repeats does not affect subunit assembly. This is in agreement with the presence of the coiled-coil region at the NH₂-terminus of COMP. The coiled-coil region has been shown to be responsible for the formation of the interchain disulfide bonds and pentamerization of the protein (34, 35). The fact that the MUT3 subunits assemble normally suggests that they can be incorporated into pentamers with the wild-type subunits and is consistent with the proposed dominant-negative effect of COMP mutations in PSACH.

COMP was originally purified from cartilage, chondrosarcoma tissues, and tendon in the presence of EDTA. During biosynthesis and in the extracellular environment, COMP is expected to exist in the presence of millimolar levels of calcium. We have kept calcium present throughout the purification of COMP to maintain calcium-dependent structures. This approach is based on the observation that removal of calcium from TSP1 results in rearrangement of intrachain disulfide bonds through a mechanism that is probably not reversible (24, 36, 37). An important property of wild-type COMP reported...
here is that COMP binds calcium ions and displays different conformations as a function of calcium concentration. This is in contrast to previous studies that failed to show any conformational changes in the presence or absence of calcium (27–30). We have shown that each subunit of COMP binds \(-9 \pm 1\) calcium ions. COMP has a compact conformation in the calcium-replete form as observed in rotary shadowing EM studies. When chelated with EDTA, COMP undergoes a conformational change into an extended form. Our observation is in accordance with the presence of the highly conserved type 3 calcium-binding domain and previous studies on TSP1, TSP3, and TSP4 (6, 7, 31–33). This serves as a molecular basis for the hypothesis that mutations in the type 3 calcium-binding repeats affect the ability of MUT3 to bind calcium and to fold correctly.

We have used sucrose gradient centrifugation, rotary shadowing EM, and limited trypsin digestion to detect conformational differences between wild-type COMP and MUT3. Although electron microscopic images of MUT3 are indistinguishable from those of COMP in the absence of calcium, MUT3 is clearly different from COMP in the calcium-replete form. The recombinant MUT3 protein appears to be less compact than wild-type COMP, with each subunit appearing more extended by electron microscopy. The greater level of asymmetry results in a decrease in the rate of sedimentation in sucrose gradients. A decrease in the rate of sedimentation is also observed in TSP1 when the molecule is treated with EDTA (31). It is interesting that MUT3 displays an intermediate conformation between the calcium-replete and calcium-depleted forms of COMP. The mutation of MUT3 disrupts the calcium-binding consensus sequence in one of the calcium-binding loops of COMP (Fig. 1). It has been shown that TSP1 binds calcium cooperatively and that nearly all calcium-binding sites have to be filled for TSP1 to fold into the final trypsin-resistant conformation (25). In this work, we have shown that at both 300 \(\mu M\) and 1.2 \(mM\) \(\text{CaCl}_2\), MUT3 does not bind as many calcium ions as wild-type COMP. The difference in the susceptibility of the two molecules to trypsin at 1.2 \(mM\) calcium reflects the mutant protein’s inability to bind the maximum number of calcium ions. These data suggest that the single amino acid deletion found in MUT3 affects the overall calcium binding and final folding of the protein.

The EM observation is supported by our limited trypsin digestion studies. At low calcium concentration, COMP and MUT3 are almost equally accessible to trypsin digestion and readily digested into small fragments. As the calcium concentration increases, COMP folds into an intermediate conformation as represented by the generation of the 50-kDa fragments. This intermediate conformation of COMP seems to be transient since COMP quickly folds into its next more compact conformation with increasing calcium concentration as illustrated by the appearance of the 63-kDa band and then the final conformation as represented by the 67-kDa bands (Fig. 5). In the case of MUT3, the intermediate conformation is predominant, and the 63-kDa band does not appear until the calcium concentration reaches the millimolar range. The 67-kDa band does not appear at all in the MUT3 digestion, even at the highest calcium concentration of 2 \(mM\), indicating that MUT3 fails to fold into the final compact calcium-replete conformation. The \(\text{NH}_2\)-terminal amino acid sequence analysis indicates that all of the major tryptic fragments begin in the same place and that the differences in their molecular masses are due to different cleavage sites at the COOH-terminal ends. According to the fragment sizes and amino acid composition, especially the ratio of alanine and phenylalanine, we postulate that at low calcium concentrations, trypsin cuts between Arg\(^{79}\) and Thr\(^{80}\), with the second cuts in the type 3 calcium-binding loops to generate the 27- and 50-kDa fragments. As the calcium concentration increases, COMP folds into more compact conformations, and these sites are protected. Instead, trypsin cuts at the end of the type 3 repeats or inside the C-globe region to generate the 63-kDa fragment. This site is protected when the calcium concentration further increases, and COMP folds into its final compact conformation. By contrast, MUT3 fails to fold into this final conformation even at the highest concentration of calcium tested. Since the conformation of COMP at high calcium concentrations probably protects a trypsic site inside the C-globe region, it is possible that the C-globe region and the type 3 repeats interact with each other and act as one structural entity. This explains the fact that some of the mutations identified in the C-globe region also cause PSACH and EDM1 (10, 11).

Based on the trypsin digestion patterns and similarities to TSP1, we can also postulate the sequence of protein folding and assembly of COMP (38). Basically, the assembly and folding start from the NH\(_2\) terminus toward the COOH terminus. In this scenario, mutations in the type 3 repeats close to the COOH terminus do not affect subunit assembly that happens at the NH\(_2\) terminus. For TSP1, subunit assembly occurs while the polypeptides are still being translated (38). Deletion of one aspartate residue between the tenth and eleventh calcium-binding loops of COMP may render one of these calcium-binding loops unable to bind calcium and to fold into its correct conformation. This may affect the folding of other parts of the protein downstream of the mutation and ultimately affect the final conformation and perhaps also the disulfide bond arrangement of the whole protein.

In this report, we have shown that deletion of a single amino acid in COMP causes it to misfold. The processing of TSP1 and other proteins in the ER indicates that conformational maturity, including that caused by proper calcium-dependent folding, is important for the transport of proteins out of the ER. For example, the bound calcium ions of the asialoglycoprotein receptor H1 subunit form an integral part of the protein structure. Depletion of calcium from the ER totally blocks H1 maturation and transport from the ER (39). In the case of TSP1, protein chaperones in the ER including BiP bind to TSP1, help in its folding, and then dissociate from TSP1 after its conformational maturation. The overall pathway of TSP1 folding is the rate-limiting step in the export of TSP1 to the Golgi (38). It is not unreasonable to assume that in the case of COMP, during its processing in the ER, some resident ER chaperones bind to it and help in its folding. The PSACH and EDM1 mutations disturb its capacity to bind calcium and to fold into its proper conformation. It is possible that the bound chaperones cannot dissociate from the misfolded proteins and that the complex is retained in the ER. However, we should point out that the retention of mutant COMP inside the ER seems to be partial and cell type-specific. In this study, we found that MUT3 is secreted by 293 cells, albeit with lower efficiency than COMP. With some PSACH mutations, it has been shown that only differentiated chondrocytes in vivo, but not adjoining tendon nor dedifferentiated chondrocytes grown in monolayer, show intracellular retention of mutant forms of COMP (20–22). The mechanism for the retention of mutant COMP in differentiated chondrocytes warrants further research.

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