Calcium Determines the Shape of Fibrillin*

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Velocity sedimentation experiments using authentic fibrillin-1 demonstrated sedimentation coefficients of $s_{20,w} = 5.1 \pm 0.1$ in the Ca$^{2+}$ form and $s_{20,w} = 6.2 \pm 0.1$ in the Ca$^{2+}$-free form. Calculations based on these results and the corresponding molecular mass predicted a shortening of fibrillin by $\sim 23\%$ and an increase in width of $\sim 13-17\%$ upon removal of Ca$^{2+}$. These observations were confirmed by analysis of Ca$^{2+}$-loaded and Ca$^{2+}$-free rotary shadowed fibrillin molecules. Analysis of recombinant fibrillin-1 subdomain rF17, consisting primarily of an array of 12 Ca$^{2+}$-binding epidermal growth factor (cbEGF)-like repeats, by analytical ultracentrifugation and rotary shadowing further confirmed Ca$^{2+}$-dependent structural changes in the tertiary structure of fibrillin-1. Based on these results, the contribution of a single cbEGF-like repeat to the length of tandem arrays is predicted to be $\sim 3$ nm in the Ca$^{2+}$ form. Ca$^{2+}$-free forms demonstrated a decrease of $20-30\%$ in length, indicating significant structural changes of these motifs when they occur in tandem. Circular dichroism measurements of rF17 in the presence and absence of Ca$^{2+}$ indicated secondary structural changes within and adjacent to the interdomain regions that connect cbEGF-like repeats. The results presented here suggest a flexible structure for the Ca$^{2+}$-free form of fibrillin which becomes stabilized, more extended, and rigid in the Ca$^{2+}$ form.

Fibrillins are integral components of extracellular supramolecular aggregates, called microfibrils (10–12 nm in diameter), which are found both in conjunction with elastic fibers and as isolated microfibrils (1, 2). Characteristic of the two highly homologous fibrillins is the mosaic composition of different types of extracellular modules (2–6). Most of the thread-like fibrillin molecule (7) is contributed by 43 epidermal growth factor (EGF)$^1$-like motifs which contain a consensus sequence for calcium binding (cb).

Calcium binding to fibrillin is now well established. It has been demonstrated with authentic fibrillin purified from cell culture medium (5), with pepsin-resistant fragments of fibrillin (8), with recombinant subdomains of fibrillin-1 (9, 10), and with synthetic peptides (11, 12). Calcium binding to fibrillin has been suggested to be important for protein-protein interaction (13), stabilization of the lateral packing of the microfibrils (14), protection of fibrillin against proteolysis (15), and maturation of a proform of fibrillin (16). The importance of calcium binding to the fibrillins is especially emphasized by the fact that mutations, predicted to disturb calcium binding, result in the pathological manifestations of the Marfan syndrome (reviewed in Ref. 17) and congenital contractual arachnodactyly (18).

The EGF-like motif is a widely used module found in more than 70 extracellular proteins (reviewed in Ref. 19). The motif consists of 40–50 amino acid residues with six highly conserved cysteine residues that form three disulfide bonds. In a subset of EGF-like modules, a characteristic pattern of amino acid residues (D/N)X(D/N)(Q/E)X$_n$(D'/N')X$_n$(Y/F); residues with an asterisk are potentially $\beta$-hydroxylated has been identified, which is responsible for calcium binding (20–22). The Ca$^{2+}$-binding site of cbEGF-like motifs has been well characterized by nuclear magnetic resonance studies of a single cbEGF-like motif of factor X (23), of a pair (numbers 32–33) of cbEGF-like motifs in fibrillin-1 (24), and by x-ray diffraction of a isolated cbEGF-like motif from human factor IX (25) and of the complex of factor VIIa with soluble tissue factor (26). From these studies, it is clear that Ca$^{2+}$ binding occurs in a NH$_2$-terminal pocket of cbEGF-like repeats between the major two-stranded $\beta$-sheet and the NH$_2$-terminal loop.

In many proteins of the extracellular matrix, cbEGF-like repeats are arranged in tandemly repeated arrays from 2 to up to 36 repeats. Examples are proteins for specification of cell fate (27–29), blood coagulation factors (reviewed in Ref. 30), anticoagulation factors (31), basement membrane proteins (32), and the fibrillins (2–6). While Ca$^{2+}$-binding sites in single cbEGF-like motifs are well understood (23–25), less information is available on how Ca$^{2+}$ binding affects the secondary and tertiary structure of tandemly repeated cbEGF-like motifs. Based on asymmetric crystallization of a cbEGF-like module, Rao and co-workers (25) suggested a Ca$^{2+}$-stabilized helical arrangement for tandemly repeated cbEGF-like motifs. Contrary to this hypothesis, nuclear magnetic resonance analyses of a covalently linked pair of cbEGF-like repeats suggested an extended and rigid conformation for tandem repeats (24).

To clarify these important issues, we investigated the effect of calcium on the shape and secondary structure of authentic fibrillin and a recombinant subdomain comprising the longest stretch of cbEGF-like repeats present in fibrillin-1. We found that in the presence of Ca$^{2+}$, the shape of fibrillin and of the recombinant subdomain was more extended compared to the Ca$^{2+}$-free forms. Additionally, circular dichroism measurements indicated subtle changes within the interdomain regions between cbEGF-like repeats. We suggest that these interdomain regions are more flexible without Ca$^{2+}$ and more rigid in the presence of Ca$^{2+}$.
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EXPERIMENTAL PROCEDURES

Protein Purification and Analytical Methods—Fibrillin was purified from fibroblast cell culture medium according to previously described methods (17). Design of recombinant fibrillin-1 subdomain rF17, comprising the longest stretch of ßEGF-like repeats and the preceding 8-cysteine motif (amino acid residues 952–1527), and purification of rF17 by chelating chromatography has been described (15). Concentrations of purified rF17 were determined in triplicate after hydrolysis (6 M HCl, 110 °C, 24 h) using an amino acid analyzer (Beckman 6300).

Velocity Sedimentation—Normal human skin fibroblasts (low passage numbers) were grown to confluency in 500-cm² cell culture flasks (Nunc). The cells were incubated with serum-free medium (Dulbecco’s modified Eagle’s medium) for 48 h. 200 ml of the serum-free medium was treated with 2 μl/ml diisopropyl fluorophosphate and passed over gelatin-Sepharose 4B (8 ml bed volume; Pharmacia) to remove fibronecin. The flow-through was concentrated to ~3 ml by ultrafiltration, dialyzed against 50 mM Tris-HCl, pH 7.5, 150 mM NaCl (TBS), and then supplemented with either 5 mM CaCl₂ or 5 mM EDTA. Aliquots (100 μl) were then pipetted on top of a 5–20% (w/v) sucrose gradient (3.6-ml total volume) buffered with TBS including either 5 mM CaCl₂ or 5 mM EDTA in Polyallomer tubes (11 × 60 mm; Beckman). Ultracentrifugation experiments were performed for 17 h at 40,000 rpm (average relative centrifugal force = 164,000 × g) at 4 °C in a Beckman L8-M ultracentrifuge using a Beckman SW60Ti rotor. After a small hole was pricked with a pin in the bottom of the tubes, 8 drop fractions were collected. Aliquots (10 μl) of the fractions were mixed with 10 μl of 2-fold concentrated SDS nonreducing sample buffer and then separated by SDS-gel electrophoresis on 4.5% (w/v) acrylamide gels (33). After transfer of the proteins to nitrocellulose membranes (Micron Separations Inc.) in 10 mM sodium borate, pH 9.2, at 0.4 A for 45 min, fibrillin-1 was visualized by a typical Western blot analysis using monoclonal antibody 201 (~10 μg/ml) and goat anti-mouse IgG horseradish peroxidase conjugate (1:2000 diluted; Bio-Rad). Monoclonal antibody 201 is specific for fibrillin-1 and does not react with fibrillin-2.2 The membranes were developed with SuperSignal™ as instructed by the manufacturer (Pierce). X-ray films (X-Omat AR; Kodak) were exposed to the membranes for a few seconds and then developed. The intensity of fibrillin-1 bands was then quantified on a Macintosh (9500/132) using the public domain NIH Image program version 1.6 (developed at the National Institutes of Health), which is available on the Internet.3 Sedimentation coefficients from sucrose gradients were calculated as described previously (34). Calculations predicting the shape of fibrillin-1 were performed according to Bloomfield et al. (35).

After purification of rF17 by chelating chromatography, the subdomain was dialyzed against TBS and then supplemented to a final concentration of either 5 mM CaCl₂ or 5 mM EDTA. The subdomain was then concentrated in concentrations of 0.55–2.9 mg/ml by analytical ultracentrifugation in a Spinco Model E centrifuge (Beckman) equipped with an optical scanner operating at 280 nm. Velocity sedimentation experiments were performed at 52,000 rpm in double sector cells at 20 °C. The absorbance scans were analyzed by the Ultrascan software (supplied by Dr. Berries Demeler).

Rotary Shadowing and Electron Microscopy—Fibrillin purified from cell culture medium was dialyzed against H₂O and then supplemented with either 2 mM CaCl₂ or 0 mM EDTA. Recombinant subdomain rF17 was dialyzed against H₂O including 2 mM CaCl₂ or 0.5 mM EDTA. Samples were diluted to a final concentration of 70% (v/v) sucrose gradient (3.6 ml total volume) buffered with TBS including either 5 mM CaCl₂ or 5 mM EDTA in Polyallomer tubes (11 × 60 mm; Beckman). Ultracentrifugation experiments were performed for 17 h at 40,000 rpm (average relative centrifugal force = 164,000 × g) at 4 °C in a Beckman L8-M ultracentrifuge using a Beckman SW60Ti rotor. After a small hole was pricked with a pin in the bottom of the tubes, 8 drop fractions were collected. Aliquots (10 μl) of the fractions were mixed with 10 μl of 2-fold concentrated SDS nonreducing sample buffer and then separated by SDS-gel electrophoresis on 4.5% (w/v) acrylamide gels (33). After transfer of the proteins to nitrocellulose membranes (Micron Separations Inc.) in 10 mM sodium borate, pH 9.2, at 0.4 A for 45 min, fibrillin-1 was visualized by a typical Western blot analysis using monoclonal antibody 201 (~10 μg/ml) and goat anti-mouse IgG horseradish peroxidase conjugate (1:2000 diluted; Bio-Rad). Monoclonal antibody 201 is specific for fibrillin-1 and does not react with fibrillin-2.2 The membranes were developed with SuperSignal™ as instructed by the manufacturer (Pierce). X-ray films (X-Omat AR; Kodak) were exposed to the membranes for a few seconds and then developed. The intensity of fibrillin-1 bands was then quantified on a Macintosh (9500/132) using the public domain NIH Image program version 1.6 (developed at the National Institutes of Health), which is available on the Internet.3 Sedimentation coefficients from sucrose gradients were calculated as described previously (34). Calculations predicting the shape of fibrillin-1 were performed according to Bloomfield et al. (35).

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Circular Dichroism Measurements—To deplete rF17 of Ca²⁺, pools of rF17 after chelating chromatography were supplemented to a final concentration of 10 mM EDTA and then dialyzed against 10 mM MOPS, pH 7.2, containing 5 g of Chelex 100 resin (Bio-Rad) per 100 ml of buffer. Alternatively, Chelex 100-treated rF17 was dialyzed against 10 mM MOPS, pH 7.2, 100 mM NaCl, 0.2 mM EDTA. Spectra from 260 to 280 nm were recorded on a Jasco J-500A instrument in the presence and absence of 2 mM CaCl₂ in a 0.1-mm cell at 25 °C. Secondary structure analysis was performed with the variable selection method (37).

RESULTS

The experiments described were designed to study the effects of calcium on the shape and secondary structure of fibrillin.

Results from velocity sedimentation experiments using fibrillin-1 were visualized by fluorescence-enhanced Western blot analysis using monoclonal antibody 201, specific for fibrillin-1 (15). The peak fractions of fibrillin-1, determined by densitometric measurements of duplicate samples, clearly differed for sedimentation in the presence of CaCl₂ (fraction 16.2) versus EDTA (fraction 13.9). These data resulted in sedimentation coefficients of 8.60 ± 0.1 S.D.) for the Ca²⁺ form and 8.20 ± 0.1 S.D.) for the Ca²⁺-free form (Table I). Predicted shapes were calculated using the experimentally determined sedimentation coefficients, estimates for partial specific volume and degree of hydration, and estimated molecular masses (39)(Table I). Based on previous data (10), we assumed that 8.5% of the total mass of fibrillin-1 is contributed by N-linked oligosaccharides. Since it is not clear whether the fibrillin-1 used was processed as has been suggested (10, 13, 38), predicted shapes were calculated for the non-processed (334 kDa) and the processed (312 kDa) form of fibrillin-1 (Table I). Upon removal of Ca²⁺ by EDTA, the relative length of fibrillin molecules is predicted to decrease by about 25%, whereas the width of the molecules is predicted to increase by 13–17%. Assuming a degree of hydration of 0.1 cm³/g, a rod-like shape, and non-processed fibrillin, the length of the molecules would be 156.1 nm in the presence of Ca²⁺ versus 117.0 nm. 

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3 Anonymous ftp site, zippy.nimh.nih.gov.
when Ca\(^{2+}\) is removed. Assuming the fibrillin used was processed, the molecules would be predicted to decrease from 142.0 nm in the Ca\(^{2+}\) form to 106.7 nm in the Ca\(^{2+}\)-free form. These results demonstrate significant structural changes of fibrillin in the presence and absence of Ca\(^{2+}\) ions.

Purified fibrillin was visualized by rotary shadowing and transmission electron microscopy in the presence of low amounts of either CaCl\(_2\) or EDTA (Fig. 2). The molecules were nicely resolved in the presence of CaCl\(_2\) and appeared as extended thread-like particles as reported previously (7) (Fig. 2A). In the presence of EDTA single particles often appeared shorter, somewhat wider and more diffuse (Fig. 2B). Length measurements resulted in 140.3 ± 14.9 nm (± S.D.; \(n = 41\)) for the Ca\(^{2+}\) form and 113.7 ± 14.9 (± S.D.; \(n = 36\)) for the Ca\(^{2+}\)-free form of fibrillin. Measurements for the width, after subtraction of 1.8 nm for the platinum coating, were 2.0 ± 0.6 nm (± S.D.; \(n = 62\)) for the Ca\(^{2+}\) form and 3.0 ± 0.8 nm (± S.D.; \(n = 56\)) for the Ca\(^{2+}\)-free form. These data corresponded with the calculations based on the sedimentation coefficients (Table I) and suggested that previous measurements of fibrillin (7) were performed using molecules in the Ca\(^{2+}\) form.

Since Ca\(^{2+}\) binds to cbEGF-like motifs, which are tandemly repeated in fibrillin-1, the structural changes observed are most likely to occur within these tandem repeats. To focus on structural changes of tandemly repeated cbEGF-like modules, we analyzed a recombinant fibrillin-1 subdomain (rF17), comprising the longest stretch of cbEGF-like motifs present in fibrillin-1 and the preceding 8-cysteine motif.

After dialysis against water including 2 mM CaCl\(_2\) or 0.5 mM EDTA, rF17 was rotary shadowed and visualized by transmission electron microscopy (Fig. 3). The representative fields clearly show that the Ca\(^{2+}\) form of rF17 adopts an extended thread-like and often straight shape (Fig. 3A), whereas the Ca\(^{2+}\)-free form of rF17 appears shorter, somewhat wider and less straight (Fig. 3B). Length measurements revealed 38.0 ± 2.7 nm (± S.D.; \(n = 100\)) for the Ca\(^{2+}\) form and 31.5 ± 3.3 nm (± S.D.; \(n = 110\)) for the Ca\(^{2+}\)-free form of rF17 (Table II). The length distribution is somewhat wider for the Ca\(^{2+}\)-free form of rF17, indicating a more variable structure compared to the Ca\(^{2+}\) form (Fig. 4). The width of rF17 after subtraction of 1.8 nm for the platinum coating was 1.8 ± 0.5 nm (± S.D.; \(n = 100\))

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**Table I**

|        | \(s_{20, w}\) | Protein mass | Degree of hydration | Prolate Length | Prolate Width | Rod Length | Rod Width | Measured\(d\) Length | Measured\(d\) Width |
|--------|--------------|--------------|---------------------|----------------|--------------|------------|------------|---------------------|---------------------|
| Ca\(^{2+}\) | 5.1 ± 0.1 | 334 | 0.1 | 160.6 | 2.2 | 156.1 | 1.8 | 140.3 ± 14.9 | 2.0 ± 0.6 |
| EDTA   | 6.2 ± 0.1 | 334 | 0.1 | 120.8 | 2.5 | 117.0 | 2.1 | 113.7 ± 14.9 | 3.0 ± 0.8 |

\(a\) Since the fibrillin-1 concentration in the sample was very low, for the calculations zero protein concentration was assumed.  
\(b\) Molecular mass of full-length and processed molecules calculated from sequence and assuming 6.5% mass from glycosylation (10).  
\(c\) Width assuming 1.8 nm of platinum coating.  
\(d\) \(n\) is number of rotary shadowed molecules measured.
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### TABLE II

| $s_{w0}$ | $m^a$ | Degree of Hydration | Prolate | Rod | Measured$^b$ |
|----------|-------|---------------------|---------|-----|--------------|
|          |       |                     | Length  | Width| Length  | Width|          |
| Ca$^{2+}$ | 3.2 ± 0.1 | 71 | 0.1 | 40.3 | 2.0 | 38.4 | 1.7 | 38.0 ± 2.7 | 1.8 ± 0.5 |
| EDTA     | 3.9 ± 0.2 | 71 | 0.1 | 28.5 | 2.4 | 26.6 | 2.0 | 31.5 ± 3.3 | 2.7 ± 0.7 |

$^a$ Molecular mass calculated from sequence and the change in mobility in SDS-polyacrylamide gel electrophoresis upon treatment with N-glycanase (15). The mass for glycosylation amounts to 10%.

$^b$ Width assuming 1.8 nm of platinum coating.

$^c$ $n$ is number of rotary shadowed molecules measured. The partial specific volume used is 0.71 cm$^3$/g.

for the Ca$^{2+}$ form and 2.7 ± 0.7 nm (± S.D.; $n = 100$) for the Ca$^{2+}$-free form.

The sedimentation coefficients for rF17 in the presence of CaCl$_2$ or EDTA were determined by analytical ultracentrifugation (Fig. 5). Extrapolated to zero protein concentration, we determined coefficients of $s_{w0} = 3.2 ± 0.1$ (± S.D.) for the Ca$^{2+}$ form and $s_{w0} = 3.9 ± 0.2$ (± S.D.) for the Ca$^{2+}$-free form of rF17 (Table II). No di- or multimerization of rF17 at high protein concentrations up to 2.9 mg/ml was observed. As demonstrated previously, about 10% of the total mass of rF17 originates from N-linked oligosaccharides (15), and therefore the total mass of rF17 was calculated to be 71 kDa. Using this molecular mass, the shape of rF17 was calculated for different degrees of hydration (35) (Table II). Upon removal of Ca$^{2+}$ by EDTA the predicted length of rF17 decreased by about 29–32% and the predicted width increased by about 18–22%, similar to observations with authentic fibrillin. Assuming 0.1 cm$^3$/g degree of hydration and a rod-shaped form, the length of rF17 is predicted to be 38.4 nm in the Ca$^{2+}$ form and 26.6 nm in the Ca$^{2+}$-free form, which is in agreement with measurements of rotary shadowed particles of rF17 (Table II). Assuming the 8-cysteine motif is globular with a diameter of 2–3 nm, the length contribution of a single cbEGF-like motif to a tandem array is about 3.0 nm when Ca$^{2+}$ is bound and may decrease to as little as 2.0 nm upon removal of Ca$^{2+}$.

Far-UV circular dichroism spectra for rF17 in the presence and absence of Ca$^{2+}$ are shown in Fig. 6. The overall appearance of the two spectra are different, suggesting electronic or conformational changes as a result of coordination of Ca$^{2+}$. The Ca$^{2+}$-free form showed a maximum at 185 nm ($\Delta \varepsilon = -0.45$) and a minimum at 202 nm ($\Delta \varepsilon = -3.23$). When Ca$^{2+}$ ions bound to rF17, there was a decrease of $\Delta \varepsilon$ between 180 and 195 nm with a minimum at 191 nm ($\Delta \varepsilon = -3.0$) and an increase of $\Delta \varepsilon$ between 196 and 216 nm with a maximum at 202 nm ($\Delta \varepsilon = -1.27$). When calculating the relative amounts of secondary structure by the variable selection method (Table III), as expected, relatively low amounts of $\alpha$-helix (8%), and high amounts of $\beta$-sheets (25–26%) and $\beta$-turns (24–26%) were observed. Only small differences in secondary structure between the Ca$^{2+}$ form and the Ca$^{2+}$-free form were observed. These small differences were between $\beta$-sheets (1–2%), $\beta$-turns (2%), and “other” structural elements (4%). These data indicate that secondary structural changes cannot account for the observed differences in length and that secondary structural changes likely also occur in interdomain regions between cbEGF-like repeats.

### DISCUSSION

In this study, we demonstrated significant differences in the tertiary structure of fibrillin in the presence and absence of Ca$^{2+}$. As determined by velocity sedimentation experiments and confirmed by measurements of rotary shadowed molecules, fibrillin shrinks in length by about 25% and becomes wider by about 13–17% when Ca$^{2+}$ is removed from the molecule. Since Ca$^{2+}$ binding to fibrillin is mediated by cbEGF-like modules, which are arranged in arrays of variable numbers of repeats, the structural changes observed must be associated with this type of motif or with its tandem arrangement. In order to substantiate these results, we analyzed a small (71 kDa) recombinant subdomain of fibrillin-1 (rF17), consisting primarily of cbEGF-like repeats (12) plus the preceding 8-cysteine motif, by analytical ultracentrifugation and rotary shadowing in the presence and absence of Ca$^{2+}$. Based on the results presented here, we calculated that a single cbEGF-like repeat contributes about 3 nm in the Ca$^{2+}$ form to the length of fibrillin. In the Ca$^{2+}$-free form, this length may be decreased by 20–30%.

The best characterized interaction of Ca$^{2+}$ with cbEGF-like motifs has been demonstrated with single cbEGF-repeats from blood coagulation factors IX and X (23, 25). The Ca$^{2+}$-binding site has been described in an amino-terminal pocket with seven...
liganding oxygen atoms in a pentagonal bipyramidal geometry (23, 25). The NH₂-terminal amino acid residues are linked by a Ca²⁺ ion to the β-turn in the main β-sheet. Upon removal of Ca²⁺, only locally restricted structural changes occur at the NH₂ terminus and at the β-turn on top of the main β-sheet (23). We compared dimensions of Ca²⁺-loaded and Ca²⁺-free forms of published structures of human factor IX (25, 39) and bovine factor X (23, 40) using Insight II (version 2.3.0; Biosym Technologies). Two solution structures of factor X determined by nuclear magnetic resonance were compared and the solution structure of the Ca²⁺-free form of factor IX (39) was compared with the crystal structure of the Ca²⁺ form (25). Depending on the residues chosen for measurements, in factor X the length of the cbEGF-like repeat is maximally ~6% shorter in the Ca²⁺-free form. In factor IX the repeat is maximally ~11% shorter in the Ca²⁺-free form. Thus, structural changes within single cbEGF-like repeats cannot account solely for the shortening effect of tandemly repeated cbEGF-like motifs observed in this study. Other changes, most likely in the connecting region between adjacent repeats, must occur. This suggestion was substantiated by circular dichroism measurements of rF17 in the presence and absence of Ca²⁺ which demonstrated only minor changes in secondary structure.

Based on an asymmetric crystallization of two units of a cbEGF-like repeat from human factor IX, which are not covalently linked to each other, it was suggested that tandemly arranged cbEGF-like repeats adopt a tightly wound helical arrangement stabilized by Ca²⁺ (25). In such a model, a single cbEGF-like repeat would only contribute about 1.5 nm to the length of a tandem array. The results presented in our study contradict this model.

Recently, the solution structure of a pair of cbEGF-like repeats of fibrillin-1 (numbers 32 and 33) in the Ca²⁺ form was reported, suggesting that Ca²⁺ rigidifies the interdomain region between the two cbEGF-like repeats (24). The two repeats were in a nearly straight orientation along the long axes with only a slight tilt angle of about 18°. A model for repeat numbers 32–36 predicted a length of 14.5 nm, suggesting that a single cbEGF-like repeat contributes about 2.9 nm to the length of the tandem array (24). These suggestions are in good agreement with the results presented in this study.

No structural data are available for the Ca²⁺-free form of tandemly repeated cbEGF-like motifs. Condensation upon removal of Ca²⁺, as observed for fibrillin-1 and recombinant rF17, could be explained by either more flexibility in the interdomain region or, alternatively, by adopting a certain fixed tilt angle between adjacent cbEGF-like repeats. Some evidence for more flexibility in the interdomain region comes from single repeats of clotting factors. In the Ca²⁺-free form, the amino termini were reported to be poorly defined (39–41), which was attributed to physical mobility within this region (40, 41). Since Ca²⁺ connects the NH₂ terminus (linking region between to adjacent repeats) to the major β-sheet of the repeats, it is conceivable that upon removal of Ca²⁺, the linking domain becomes more flexible. If the interdomain region is indeed more flexible in the absence of Ca²⁺ one would expect wider length distributions of tandem arrays in the Ca²⁺-free form compared to the Ca²⁺-form with the “locked” extended conformation. Length distributions of rotary shadowed rF17 in the presence and absence of Ca²⁺ in fact showed a somewhat wider distribution for the Ca²⁺-free form. However, in sedimentation experiments of authentic fibrillin-1, approximately the same band width was observed for the Ca²⁺-loaded and the Ca²⁺-free form.

To reduce the apparent length of a single motif within a tandem array from about 3 nm in the extended form to as little as 2 nm, a tilt angle of about 96° would be required between adjacent repeats. Interestingly, x-ray diffraction data from porcine factor IXa, which contains two tandemly repeated EGF-like repeats (one NH₂-terminal Ca²⁺-binding and one COOH-terminal non-calcium binding repeat) demonstrated a tilt

![Fig. 5. Velocity sedimentation of recombinant fibrillin-1 subdomain rF17 in the presence of 5 mM CaCl₂ (□) or 5 mM EDTA (■) at 20 °C. Sedimentation coefficients were corrected for water.](image)

![Fig. 6. Far UV circular dichroism spectra of recombinant fibrillin-1 subdomain rF17 in the presence (+Ca²⁺) and absence (−Ca²⁺) of 2 mM CaCl₂ at 25 °C. Δε per residue is shown as a function of wavelength. The experiment was performed with rF17 (1.7 mg/ml) in Chelex-treated 10 mM MOPS, pH 7.2, without salt. The experiment was repeated slightly modified using rF17 (1.8 mg/ml) in 10 mM MOPS, pH 7.2, 100 mM NaCl, and 0.2 mM EDTA with identical results.](image)

| Secondary structure analysis from circular dichroism spectra of rF17 in presence and absence of Ca²⁺ |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                | α-Helix | Antiparallel β | Parallel β | Turn | Other | Total |
| Ca²⁺ | 0.08 | 0.20 | 0.05 | 0.24 | 0.45 | 1.02⁺ |
| EDTA | 0.08 | 0.22 | 0.04 | 0.26 | 0.41 | 1.01⁺ |

*Values were calculated using the variable selection method (37).
angle of 110° between the repeats (42). In clotting factors, the interdomain region between the two EGF-like repeats cannot be stabilized by Ca\(^{2+}\) since the COOH-terminal repeat is not of the Ca\(^{2+}\)-binding type and the Ca\(^{2+}\)-binding site of the NH\(_2\)-terminal repeat is distal from the interdomain region.

EGF-like motifs occur in at least 70 different proteins with a wide variety of biological functions. They often occur in randomly repeated arrays (19). These repeated arrays can consist of non-calcium binding EGF-like repeats as found for example in the tenascin family (43, 44) or of cbEGF-like repeats as in Drosophila Notch (27), the fibrilins (32, 45, 46), or the fibrillins (2–6). Why was Ca\(^{2+}\) binding to EGF-like motifs invented? Tandem arrays of non-calcium binding EGF-like repeats may serve as flexible hinge-like domains in modular proteins, whereas in arrays of cbEGF-like motifs, Ca\(^{2+}\) binding will stabilize an extended and rigid conformation that might be necessary to maintain the correct tertiary structure for certain protein-protein interactions or for assembly of supramolecular structures. We reported previously that cbEGF-like repeats in fibrillin-1 are protected against proteolysis by Ca\(^{2+}\) (15). Tandem stretches of cbEGF-like repeats may provide this protection to proteins which are supposed to form stable structures.

More than 50 mutations in fibrillin-1 give rise to the Marfan syndrome, a connective tissue disorder with skeletal, cardiovascular, and ocular symptoms (17). The majority of these mutations are predicted to disturb Ca\(^{2+}\) binding to cbEGF-like motifs. Structural changes upon removal of Ca\(^{2+}\), as demonstrated in this study, may resemble local structural changes in mutated fibrillin-1. The interdomain region NH\(_2\)-terminal to the cbEGF-like repeat harboring the mutation may be more flexible or adopt a certain angle different from the Ca\(^{2+}\)-loaded form. These structural changes might then disturb protein interactions or supramolecular assembly processes, or expose certain regions to proteolytic degradation. These types of interference with biological functions of fibrillin might in turn lead to the progressive nature of the disorder.

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