

## The Disulfide Bonding Pattern in Ficolin Multimers\*

Received for publication, September 24, 2003, and in revised form, November 20, 2003  
Published, JBC Papers in Press, December 1, 2003, DOI 10.1074/jbc.M310555200

Tomoo Ohashi and Harold P. Erickson‡

From the Department of Cell Biology, Duke University Medical Center, Durham, North Carolina 27710

**Ficolin is a plasma lectin, consisting of a short N-terminal multimerization domain, a middle collagen domain, and a C-terminal fibrinogen-like domain. The collagen domains assemble the subunits into trimers, and the N-terminal domain assembles four trimers into 12-mers. Two cysteine residues in the N-terminal domain are thought to mediate multimerization by disulfide bonding. We have generated three mutants of ficolin  $\alpha$  in which the N-terminal cysteines were substituted by serines (Cys<sup>4</sup>, Cys<sup>24</sup>, and Cys<sup>4</sup>/Cys<sup>24</sup>). The N-terminal cysteine mutants were produced in a mammalian cell expression system, purified by affinity chromatography, and analyzed under nonreducing conditions to resolve the multimer structure of the native protein and under denaturing conditions to resolve the disulfide-linked structure. Glycerol gradient sedimentation and electron microscopy in nonreducing conditions showed that plasma and recombinant wild-type protein formed 12-mers. The Cys<sup>4</sup> mutant also formed 12-mers, but Cys<sup>24</sup> and Cys<sup>4</sup>/Cys<sup>24</sup> mutants formed only trimers. This means that protein interfaces containing Cys<sup>4</sup> are stable as noncovalent protein-protein interactions and do not require disulfides, whereas those containing Cys<sup>24</sup>-Cys<sup>24</sup> require the disulfides for stability. Proteins were also analyzed by nonreducing SDS-PAGE to show the covalent structure under denaturing conditions. Wild-type ficolin was covalently linked into 12-mers, whereas elimination of either Cys<sup>4</sup> or Cys<sup>24</sup> gave dimers and monomers. We present a model in which symmetric Cys<sup>24</sup>-Cys<sup>24</sup> disulfide bonds between trimers are the basis for multimerization. The model may also be relevant to collectin multimers.**

In the past decade, many ficolin family proteins have been discovered in several species including pig (ficolins  $\alpha$  and  $\beta$ ) (1), human (ficolins H, L, and M) (2–6), mouse (ficolins A and B) (7, 8), and frog (ficolins 1–4) (9). Ficolin-like proteins have been reported in invertebrates such as horseshoe crab and the solitary ascidian (10, 11). Tachylectin (ficolin-like protein in horseshoe crab) lacks a collagen domain, and solitary ascidian ficolin has only a very short collagen domain (five Gly-X-Y repeats). Ficolin family proteins are functionally very similar to collectin (collagenous lectin) family proteins such as mannose-binding protein (MBP),<sup>1</sup> conglutinin, collectin-43 (CL-43), and surfactant proteins A and D (SP-A and SP-D) (12–14). Both families

of proteins have a lectin activity that is important for binding to the surface of microbes, which may contribute to innate host defense by enhancing the opsonic activity (3, 12–16). MBP and ficolins L and H seem to activate the complement pathway by associating with serine proteases (12, 13, 17, 18). Ficolins and collectins also show similar tissue distribution. MBP, conglutinin, and CL-43 are serum proteins produced by the liver (12, 13). Ficolins  $\alpha$ , L, H, and A have been purified from plasma, and their expression has been confirmed in the liver (2, 3, 8). SP-A and SP-D are expressed in the lung, as are ficolins  $\alpha$ , M, and H (8, 12–14).

In addition to similarities of function and tissue distribution, ficolin and collectin family proteins have structural similarities. Ficolins and collectins consist of a short N-terminal domain, which may be used for multimerization, a middle collagen domain, and a C-terminal globular domain that binds carbohydrate (19–26). This structure is also similar to complement protein C1q (21). The primary difference between ficolins and collectins is that the C-terminal globular domain of ficolin is a fibrinogen-like (fbg) domain, whereas that of collectins is a carbohydrate recognition domain (12–14, 26). In addition, collectin family proteins have a short  $\alpha$ -helical neck region between the collagen and C-terminal domains, whereas ficolins lack this region. The  $\alpha$ -helical neck regions of collectins seem to be important for trimerization (27–30). Both families of proteins form trimer-based multimers; CL-43 and MBP-C form trimers as the native form, whereas ficolins, conglutinin, MBP-A, and SP-D form tetramers (12-mers), and SP-A and C1q form hexamers (18-mers) (19–26, 31, 32). In some cases, larger multimers have been found for ficolin (26), MBP (25), and SP-D (19). Collectin trimers are linked to each other by disulfide bonds in the N-terminal domain (32–34). Although there is no obvious sequence similarity between the N termini of collectins and ficolins, ficolins also have cysteines that appear to mediate multimerization by disulfide bonding.

It is generally accepted that the cysteines in the N-terminal domain mediate multimerization, but the connectivity pattern has not been completely determined for either collectins or ficolins. In the present study, we have used site-directed mutagenesis to determine how the two cysteine residues in the N terminus of ficolin  $\alpha$ , Cys<sup>4</sup> and Cys<sup>24</sup>, contribute to multimerization. The pattern of connectivity we determined may be relevant to collectins as well as to ficolins.

### EXPERIMENTAL PROCEDURES

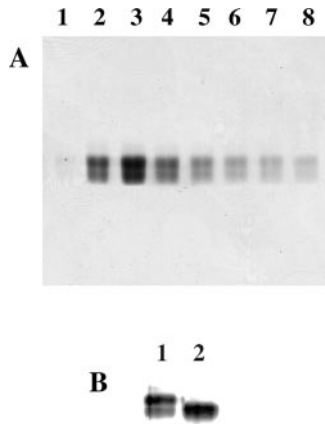
**Mammalian Cell Expression Protein**—Ficolin  $\alpha$  cDNA (pSVFC $\alpha$ ) (1), kindly provided by Hidenori Ichijo (Cancer Institute, Japan), was used as template for site-directed mutagenesis (Stratagene). Two sets of oligonucleotides (5'-CCCTCGACACCTCTCCAGAGGTCAAG-3' and 5'-CCATCTCCGAGGCTCCCGGGGCTGCCTGG-3' and their reverse complements) were designed for generating the Cys<sup>4</sup>, Cys<sup>24</sup>, and Cys<sup>4</sup>/Cys<sup>24</sup> double mutants. The mutated cDNAs were cloned into the pEE14

\* This work was supported by National Institutes of Health Grants CA47056. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Cell Biology, Box 3709, Duke University Medical Center, Durham, NC 27710. Tel.: 919-684-6385; Fax: 919-684-8090; E-mail: H.Erickson@cellbio.duke.edu.

<sup>1</sup> The abbreviations used are: MBP, mannose-binding protein; CL-43,

collectin-43; DTT, dithiothreitol; fbg, fibrinogen-like; SP-A, surfactant protein A; SP-D, surfactant protein D; TBS, Tris-buffered saline.



**FIG. 1. GlcNAc affinity purification of recombinant ficolin and N-linked glycosylated ficolin.** A, A GlcNAc column was loaded with conditioned medium from cells transfected with wild-type ficolin and eluted with 150 mM GlcNAc. Samples from each fraction were applied to SDS-PAGE (10%) under reducing conditions. The gel was stained with Coomassie Blue. Lane numbers correspond to eluted fractions. B, purified ficolin before (lane 1) and after digestion with *N*-glycosidase F analyzed by Western blotting after SDS-PAGE (12%) under reducing conditions.

expression vector with the glutamine synthetase gene as a selectable marker (35). The expression vector was transfected into Chinese hamster ovary K1 cells, using Lipofectamine (Invitrogen). The transfected cells were cultured with  $\alpha$ -minimal essential medium without glutamine (BioWhittaker) containing 10% dialyzed fetal bovine serum (Invitrogen) and selected in the presence of a glutamine synthetase inhibitor, L-methionine sulfoximine (Sigma). The highest expressing clones were identified by Western blot. The conditioned media from the clones were collected, passed through a GlcNAc-agarose column (Sigma), and eluted with 150 mM GlcNAc in 20 mM Tris buffer containing 150 mM NaCl, pH 8.0 (TBS). However, some mutants did not bind to the GlcNAc column. These inactive mutants were purified with an antibody affinity column as reported previously (26). SDS-PAGE was carried out using standard techniques.

**Western Blots**—Proteins were transferred from the SDS gel to polyvinylidene difluoride membrane (Millipore) using a semidry electroblotter (Multiphor II; Amersham Biosciences). The membrane was incubated with the polyclonal antibody Fica4325 (26) at a dilution of 1:2,000 and then with horseradish peroxidase-conjugated secondary antibody at a dilution of 1:1,000 (BIOSSOURCE) and stained with diaminobenzidine,  $H_2O_2$ , and  $NiCl_2$ .

**Estimation of Sedimentation Coefficient and Electron Microscopy**—Sedimentation coefficients were estimated by glycerol gradient sedimentation. The samples were sedimented at 20 °C on 15–40% glycerol gradients in 0.2 M ammonium bicarbonate at 38,000 rpm for 16 h in a Beckman SW-55.1 rotor. The glycerol gradients were calibrated with standard proteins of known *s* values (catalase, 11.3 S; aldolase, 7.3 S; bovine serum albumin, 4.6 S; chymotrypsinogen, 2.6 S).

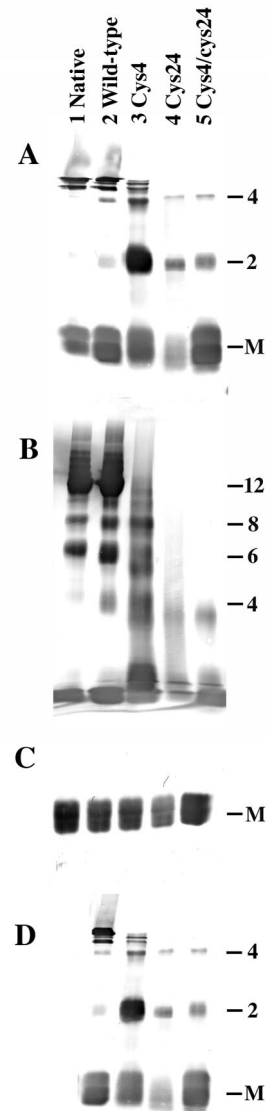
For rotary shadowing, purified ficolin from a glycerol gradient was sprayed onto freshly cleaved mica, dried under vacuum, and rotary-shadowed with platinum (36).

**Reduction, Reoxidation, and Trypsin Treatment**—Recombinant wild-type ficolin at 0.5 mg/ml was reduced with 50 mM dithiothreitol (DTT) for 1 h at room temperature. For reoxidation, the reduced proteins were dialyzed against TBS containing 1 mM reduced glutathione and 0.3 mM oxidized glutathione for 24–36 h at room temperature. In order to block free cysteine, some reduced samples were dialyzed against TBS containing 10 mM iodoacetamide for 24–36 h at room temperature. Samples were further dialyzed against TBS for 24–36 h at room temperature to remove reagents. For estimation of the sedimentation coefficient of the reduced ficolin, 1 mM DTT was added to the glycerol gradient to prevent reoxidation.

Recombinant wild-type ficolin at 0.5 mg/ml was digested with trypsin at 0.1 mg/ml for 2 h at room temperature, and the digestion was quenched with 2 mM phenylmethylsulfonyl fluoride. Protease digestion is often used to evaluate the stability of a collagen triple-helical structure (37).

## RESULTS

The recombinant wild-type ficolin was purified by GlcNAc affinity chromatography (Fig. 1A). Less than 10% of the ficolin



**FIG. 2. The covalent structure of wild-type ficolin and Cys mutants analyzed by nonreducing SDS-PAGE.** Ficolin samples were analyzed by 10% (A) and 5% (B) SDS-PAGE under nonreducing conditions and 10% SDS-PAGE under reducing conditions (C) followed by Western blotting with polyclonal antibody Fica4325. The recombinant ficolin and mutants in conditioned media were also analyzed by 10% SDS-PAGE under nonreducing conditions followed by Western blotting (D). Lane 1, plasma ficolin purified from pig. Lane 2, recombinant wild-type ficolin. Lane 3, Cys<sup>4</sup> mutant. Lane 4, Cys<sup>24</sup> mutant. Lane 5, Cys<sup>4</sup>/Cys<sup>24</sup> mutant. M, monomer; 2, 4, 6, 8, and 12, positions for different multimers based on independent molecular weight standards.

in the conditioned medium bound to the GlcNAc column, so this purification selected for the small fraction of active protein. Unlike in plasma ficolin purification (26), no obvious contaminating protein was detected in this one-step preparation. We did not detect any ficolin in fetal bovine serum by GlcNAc affinity chromatography and Western blot prior to this study, suggesting that the fetal serum may have low levels of ficolin. The recombinant ficolin always showed doublets in SDS-PAGE and on Western blots. Treatment with *N*-glycosidase indicated that the upper band was *N*-glycosylated, whereas the lower band was not (Fig. 1B). The Cys<sup>4</sup> mutants were also purified with a GlcNAc column. However, Cys<sup>24</sup> and Cys<sup>4</sup>/Cys<sup>24</sup> mutants did not bind to the GlcNAc column. They were purified by antibody affinity chromatography as reported previously (26).

Fig. 2 shows purified ficolin on a Western blot under nonreducing conditions. The plasma and recombinant wild-type ficolin

TABLE I  
Estimated molecular masses for recombinant ficolins

Protein	Molecular mass	<i>s</i>	$s_{\max}/s^a$
	<i>kDa</i>		
Native ficolin <sup>b</sup>	420 (12-mer)	12	1.7
Recombinant ficolin	420 (12-mer)	11.7	1.7
Cys <sup>4</sup> -substituted mutant	420 (12-mer)	11.3	1.8
Cys <sup>24</sup> -substituted mutant	105 (trimer)	4.5	1.8
Cys <sup>4</sup> /Cys <sup>24</sup> -substituted mutant	105 (trimer)	4.7	1.7
Reduced ficolin	105 (trimer)	4.6	1.7
Reoxidized ficolin	420 (12-mer)	11.3	1.8
Trypsin-digested ficolin	420 (12-mer)	11.7	1.7

<sup>a</sup> The ratio of maximum sedimentation coefficient, calculated for an unhydrated sphere of protein of the same mass, to the measured sedimentation coefficient.

<sup>b</sup> The molecular weight for native ficolin has been reported previously (26).

lin migrated mainly as monomers and 12-mers with a small fraction migrating as tetramers, hexamers, and 8-mers (Fig. 2, lanes 1 and 2). This demonstrates that the recombinant wild-type ficolin is covalently cross-linked by disulfide bonds, but the presence of smaller multimers suggests that some of the interchain disulfide linkages are not formed, as found previously for plasma ficolin (26). A small fraction of protein migrated above 12-mers, suggesting that ficolin is able to form covalently linked larger multimers. The Cys<sup>4</sup> mutant migrated primarily as monomers and dimers, with a small and decreasing fraction of higher multimers, up to some 12-mers (Fig. 2, lane 3). Some of the intermediate multimers ran in different positions from those of the wild-type protein. The Cys<sup>24</sup> mutants showed monomeric and dimeric forms and a small fraction of tetramers (Fig. 2, lane 4). The Cys<sup>4</sup>/Cys<sup>24</sup> mutant showed a similar pattern but with an apparently much larger monomeric pool (Fig. 2, lane 5). Note that the minor bands described here are mostly invisible on Coomassie Blue-stained SDS gels.

Because only 10% of the recombinant wild-type ficolin bound to the GlcNAc column, we were concerned that this column might be selecting for disulfide-bonded multimers. We therefore checked the disulfide bonding pattern of protein in conditioned media by Western blotting (Fig. 2D). For both the wild-type and mutant proteins, the pattern was identical to that of the proteins purified by GlcNAc or antibody affinity chromatography. This suggests that the lack of GlcNAc binding of the recombinant wild-type ficolin is not due to incomplete disulfide bond multimerization.

Samples were analyzed by glycerol gradient sedimentation to determine the sedimentation coefficients. Results for all proteins are given in Table I, and individual examples are shown in Fig. 3. Purified recombinant wild-type ficolin sedimented as a sharp peak at 11.7 S (Fig. 3A) the same as plasma ficolin 12-mers (note that the majority of plasma ficolin forms 24-mers and sediments at 19.5 S) (26). The Cys<sup>4</sup> mutant showed a similar *s* value of 11.3, suggesting that it forms 12-mers despite the altered disulfide bond formation (Fig. 2). On the other hand, the Cys<sup>24</sup> mutant sedimented at 4.5 S, suggesting that it forms only trimers. The Cys<sup>4</sup>/Cys<sup>24</sup> mutant sedimented at 4.7 S, essentially the same as Cys<sup>24</sup> (Fig. 3B). In Table I, we have calculated an  $s_{\max}/s$  for each protein based on the above size estimate. They all have the same value of about 1.7, characteristic of a moderately elongated protein (38).

Reduced and reoxidized samples were also analyzed by glycerol gradient sedimentation (Fig. 4). Reduced ficolin sedimented at 4.6 S (Fig. 4A). Samples from the gradient ran as monomers on nonreducing SDS-PAGE, which was expected, since the gradient contained DTT. To determine the extent of reduction, we treated a sample with iodoacetamide and dialyzed away the DTT. This sample migrated as monomers on

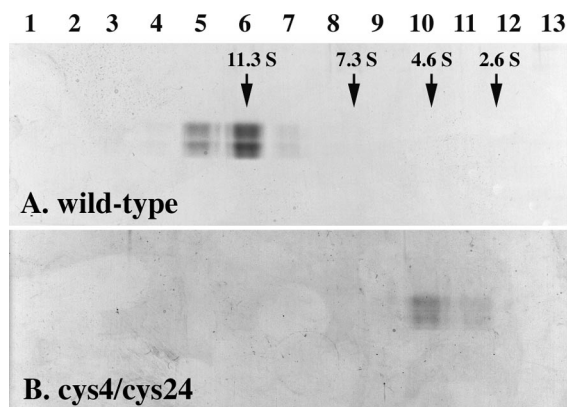


FIG. 3. Glycerol gradient sedimentation of purified ficolin. Fractions from the gradients were analyzed by SDS-PAGE (10%) under reducing conditions. A, recombinant wild-type ficolin. B, Cys<sup>4</sup>/Cys<sup>24</sup> mutant. Lane numbers correspond to gradient fraction numbers. The positions of standard proteins (from the left, catalase (11.3 S), aldolase (7.3 S), bovine serum albumin (4.6 S), and chymotrypsinogen (2.6 S)) are indicated by the arrows.

SDS-PAGE (data not shown), indicating that the intersubunit disulfides were fully reduced.

The multimeric structure could be fully restored by reoxidizing the reduced ficolin with a mixture of reduced and oxidized glutathione. The reoxidized ficolin sedimented at 11.3 S and migrated as 12-mers and monomers in SDS-PAGE under non-reducing conditions (Fig. 4B), the same as wild-type ficolin (Fig. 4C).

We also examined the effect of mild trypsin digestion on ficolin structure. The trypsin-digested ficolin sedimented at 11.7 S and migrated as monomers and dimers in SDS-PAGE (Fig. 4D). This is the same as the Cys<sup>4</sup> mutants (Fig. 2 and Table I), which suggests that trypsin digestion eliminates the Cys<sup>4</sup>-mediated disulfide linkages. Trypsin probably digests the carboxyl side of Lys<sup>8</sup>, because the digestion produced no obvious molecular weight shift in SDS-PAGE under reducing conditions (data not shown).

Electron microscopy of rotary-shadowed recombinant wild-type ficolin showed parachute-like structures (Fig. 5A), the same as plasma ficolin 12-mers reported previously (26). The small glob at the bottom of the parachute consists of the associated N-terminal domains, and the parachute itself contains the four trimers of the C-terminal fbg domains. The thin, elongated collagen domains between the N- and C-terminal domains are mostly invisible in rotary-shadowed images. Approximately 9% of molecules (35 of 396) showed disrupted parachute-like structures (Fig. 5F). Larger multimers that seemed linked at the N terminus were infrequently seen (<1%), and some showed bouquet-like structures (Fig. 5G). These larger multimers linked at the N terminus had not been seen in plasma ficolin (26), suggesting that it may be due to the overexpression of ficolin in the mammalian cell expression system. On the contrary, 24-mers linked at the C-terminal domains, which were seen in plasma ficolin (26), were rarely observed in the recombinant protein (Fig. 5H). This result is consistent with the estimated sedimentation coefficients (Table I).

The Cys<sup>4</sup> mutant showed parachute-like structures identical to wild-type ficolin (Fig. 5B), confirming the suggestion from sedimentation that it forms normal 12-mers. The Cys<sup>4</sup>/Cys<sup>24</sup> mutant showed lollipop-like structures (Fig. 5C), consistent with individual trimers. In these trimeric forms, the collagen domains are clearly seen. The N-terminal globs are almost invisible, although in some cases they appear as tiny globs (Fig. 5I). We were unable to visualize the Cys<sup>24</sup> mutant

FIG. 4. Glycerol gradient sedimentation of reduced, reoxidized, and trypsin-digested ficolin. A, reduced ficolin; B, reoxidized ficolin; C, recombinant wild-type ficolin; D, trypsin-digested ficolin. Lane numbers correspond to gradient fraction numbers. The positions of standard proteins (bovine serum albumin (A) and catalase (B–D)) are indicated by arrows.

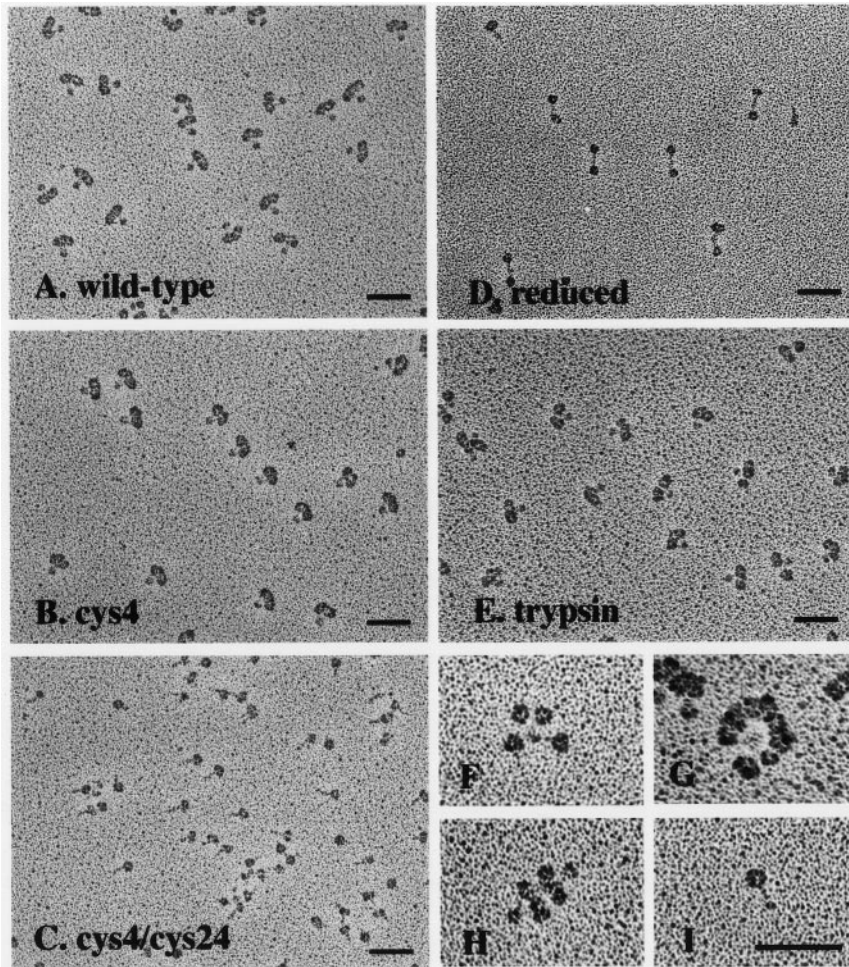
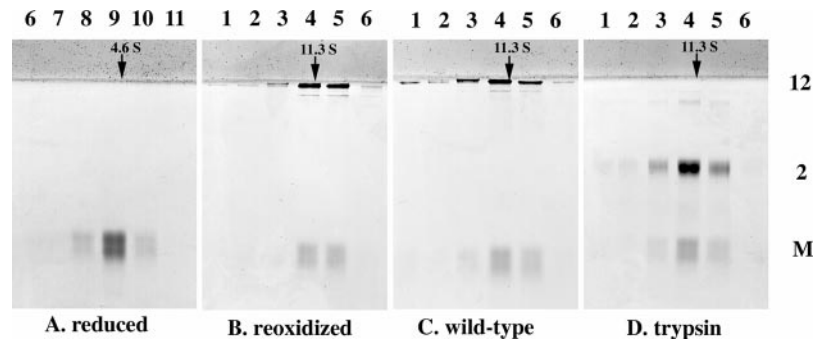


FIG. 5. Electron micrographs of rotary-shadowed recombinant ficolin. The recombinant wild-type ficolin (A) and Cys<sup>4</sup> mutant (B) show a tetrameric parachute-like structure (12-mer), the same as plasma ficolin 12-mers (26). The recombinant wild-type ficolin also showed occasional disrupted parachute-like structure (F), bouquet-like structure (G), and 24-mers (H). The Cys<sup>4</sup>/Cys<sup>24</sup> mutant showed a lollipop-like structure corresponding to trimers (C and I). Reduced ficolin (D) showed dumbbell-like or V-shaped structures that we interpret as hexamers. Some reduced molecules appeared as trimeric lollipop-like structures, the same as Cys<sup>4</sup>/Cys<sup>24</sup>. Trypsin-digested ficolin (E) showed tetrameric parachute-like structures, the same as wild-type ficolin and the Cys<sup>4</sup> mutant. Bars, 50 nm.

by EM, due to the low concentration of purified protein. These EM images agree with the multimeric structures estimated in Table I.

Electron microscopy of the reduced ficolin showed dumbbell-like or V-shaped structures (Fig. 5D), which we interpret as being two trimers connected at their N-terminal domains. Some proteins also revealed trimeric lollipop-like structures similar to the Cys<sup>4</sup>/Cys<sup>24</sup> mutants. Interestingly, the estimated sedimentation coefficient 4.6 S of the reduced ficolin is identical to that of the Cys<sup>4</sup>/Cys<sup>24</sup> substitute mutants that showed a trimeric form in EM. We suggest that the reduced ficolin shows a weak tendency to associate at the N-terminal domain but that this is blocked by the hydrostatic pressure during sedimentation. When the pressure is reduced following sedimentation, the trimers reassociate. This effect has been reported in gradient sedimentation of tubulin (39) and FtsZ (40). The reoxidized and trypsin-digested ficolin showed parachute-like structures (Fig. 5E), the same as recombinant wild-type ficolin (Fig. 5, A and B).

We also attempted to measure the number of free sulfhydryls in the recombinant wild-type ficolin by DTNB assay (41). Free cysteine and fibronectin type III domains 7–10 (FN7–10) (42) were used as positive controls. FN7–10 has a single sulfhydryl, which was detectable in the presence of urea or guanidine HCl, as reported previously (43). On the other hand, we did not detect any free sulfhydryl in the recombinant wild-type ficolin, even in the presence of urea or guanidine HCl (data not shown). These measurements were able to detect sulfhydryls at a concentration of 2–3  $\mu$ M, so at the protein concentrations we had available we could detect one free sulfhydryl per subunit, but we would not have detected ~2–4 free sulfhydryls per 12-mer.

#### DISCUSSION

A small fraction of recombinant wild-type and Cys<sup>4</sup> mutant ficolin bound the GlcNAc column, but the Cys<sup>24</sup> and Cys<sup>4</sup>/Cys<sup>24</sup> mutants did not bind at all. Since the inactive mutants are trimers, it seems that efficient binding to the GlcNAc column



disulfide with Cys<sup>24</sup> and that this may block disulfide bonding of Cys<sup>4</sup> of the same subunit.

The lack of symmetry in disulfide bonding within the trimer has a well established precedent in CL-43 and MBP-C. These collectins form trimers but not higher multimers, and the two cysteines in each chain are fully engaged in disulfide bonds. In CL-43, there were three different disulfide links: Cys<sup>15</sup>-Cys<sup>15</sup>, Cys<sup>15</sup>-Cys<sup>20</sup>, and Cys<sup>20</sup>-Cys<sup>20</sup> (47). Wallis and Drickamer (31) deduced a similar asymmetric linkage for MBP-C (Cys<sup>11</sup>-Cys<sup>11</sup>, Cys<sup>16</sup>-Cys<sup>16</sup>, and Cys<sup>11</sup>-Cys<sup>16</sup>) and presented the important argument that one should not expect a symmetric arrangement, because the molecule does not have 3-fold symmetry. The collagen molecule has a 3-fold screw axis, in which the three chains are staggered. It is therefore not surprising to find an asymmetric bonding pattern in the N-terminal domain of collectins and ficolins, since the collagen imposes a break from rotational symmetry.

MBP-A has three cysteines in its N-terminal domain. Two of these (Cys<sup>13</sup> and Cys<sup>18</sup>) are involved in forming cross-links within a trimer, and Cys<sup>6</sup> mediates association of trimers into multimers of 1–4 trimers (32). MBP-A Cys<sup>6</sup> is thus similar to Cys<sup>24</sup> of ficolin. Only one or two of the three Cys<sup>6</sup> residues per trimer could participate in bonding between trimers. Since no free cysteine was detected, the remaining Cys<sup>6</sup> is probably bonded internally to Cys<sup>13</sup> or Cys<sup>18</sup>.

SP-A has only a single universal cysteine (Cys<sup>6</sup>) in its N-terminal domain, although about 20% of the chains have an N-terminal extension with an additional cysteine at position –1. Elhalwagi *et al.* (34) have proposed that Cys<sup>6</sup> forms disulfide bonds both within and between trimers. In their model, two of the three cysteines formed disulfides within the trimer. However, this would leave only one cysteine to form disulfide bonds between trimers, which would not form multimers larger than dimers of trimers. We would suggest that Cys<sup>6</sup> is primarily involved in symmetric bonding between trimers, similar to Cys<sup>24</sup> in our model for ficolin. This would leave one chain in each trimer without a disulfide bond, which is consistent with the published gel results (34).

The collectins and ficolins thus seem to have in common the use of a single cysteine in the N-terminal domain to cross-link the trimers into multimers. In a closed circular structure, these disulfides between trimers utilize two of the three cysteines, with the third one either unbonded or bonding to another cysteine within the trimer. In addition, most of these molecules have a second cysteine that forms disulfides within each trimer.

A remarkable feature of the ficolin multimerization is that it can be reversibly altered by reducing and reoxidizing the disulfide bonds. Whereas the structure of the trimer is maintained by the noncovalent bonding within the collagen helix, association between trimers is highly dependent on disulfide bonds. The assembly of subunits first into trimers, followed by association into multimers, which are finally stabilized by disulfides, is probably the pathway of assembly in the rough endoplasmic reticulum.

## REFERENCES

1. Ichijo, H., Hellman, U., Wernstedt, C., Gønez, L. J., Claesson-Welsh, L., Heldin, C.-H., and Miyazono, K. (1993) *J. Biol. Chem.* **268**, 14505–14513
2. Sugimoto, R., Yae, Y., Akaiwa, M., Kitajima, S., Shibata, Y., Sato, H., Hirata, J., Okochi, K., Izuhara, K., and Hamasaki, N. (1998) *J. Biol. Chem.* **273**, 20721–20727
3. Matsushita, M., Endo, Y., Taira, S., Sato, Y., Fujita, T., Ichikawa, N., Nakata, M., and Mizuochi, T. (1996) *J. Biol. Chem.* **271**, 2448–2454
4. Endo, Y., Sato, Y., Matsushita, M., and Fujita, T. (1996) *Genomics* **36**, 515–521
5. Harumiya, S., Takeda, K., Sugiura, T., Fukumoto, Y., Tachikawa, H., Miyazono, K., Fujimoto, D., and Ichijo, H. (1996) *J. Biochem. (Tokyo)* **120**, 745–751
6. Lu, J., Tay, P. N., Kon, O. L., and Reid, K. B. (1996) *Biochem. J.* **313**, 473–478
7. Fujimori, Y., Harumiya, S., Fukumoto, Y., Miura, Y., Yagasaki, K., Tachikawa, H., and Fujimoto, D. (1998) *Biochem. Biophys. Res. Commun.* **244**, 796–800
8. Ohashi, T., and Erickson, H. P. (1998) *Arch. Biochem. Biophys.* **360**, 223–232
9. Kakinuma, Y., Endo, Y., Takahashi, M., Nakata, M., Matsushita, M., Takenoshita, S., and Fujita, T. (2003) *Immunogenetics* **55**, 29–37
10. Gokudan, S., Muta, T., Tsuda, R., Koori, K., Kawahara, T., Seki, N., Mizunoe, Y., Wai, S. N., Iwanaga, S., and Kawabata, S. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 10086–10091
11. Kenjo, A., Takahashi, M., Matsushita, M., Endo, Y., Nakata, M., Mizuochi, T., and Fujita, T. (2001) *J. Biol. Chem.* **276**, 19959–19965
12. Holmskov, U. L. (2000) *APMIS Suppl.* **100**, 1–59
13. Lu, J., Teh, C., Kishore, U., and Reid, K. B. (2002) *Biochim. Biophys. Acta* **1572**, 387–400
14. Crouch, E., and Wright, J. R. (2001) *Annu. Rev. Physiol.* **63**, 521–554
15. Lu, J., Le, Y., Kon, L., Chan, J., and Lee, S. H. (1996) *Immunology* **89**, 289–294
16. Teh, C., Le, Y., Lee, S. H., and Lu, J. (2000) *Immunology* **101**, 225–232
17. Matsushita, M., Endo, Y., and Fujita, T. (2000) *J. Immunol.* **164**, 2281–2284
18. Matsushita, M., Kuraya, M., Hamasaki, N., Tsujimura, M., Shiraki, H., and Fujita, T. (2002) *J. Immunol.* **168**, 3502–3506
19. Crouch, E., Persson, A., Chang, D., and Heuser, J. (1994) *J. Biol. Chem.* **269**, 17311–17319
20. Holmskov, U., Laursen, S. B., Malhotra, R., Wiedemann, H., Timpl, R., Stuart, G. R., Tronoe, I., Madsen, P. S., Reid, B. M., and Jensenius, J. C. (1995) *Biochem. J.* **305**, 889–896
21. Knobel, H. R., Villiger, W., and Isliker, H. (1975) *Eur. J. Immunol.* **5**, 78–82
22. Lu, J., Wiedemann, H., Holmskov, U., Thiel, S., Timpl, R., and Reid, K. B. M. (1993) *Eur. J. Biochem.* **215**, 793–799
23. Voss, T., Eistetter, H., and Schafer, K. P. (1988) *J. Mol. Biol.* **201**, 219–227
24. Storgaard, P., Nielsen, E. H., Andersen, O., Skriver, E., Mortensen, H., Hojrup, P., Leslie, G., Holmskov, U., and Svehag, S. E. (1996) *Scand. J. Immunol.* **43**, 289–296
25. Lu, J. H., Thiel, S., Wiedemann, H., Timpl, R., and Reid, K. B. (1990) *J. Immunol.* **144**, 2287–2294
26. Ohashi, T., and Erickson, H. P. (1997) *J. Biol. Chem.* **272**, 14220–14226
27. Hakansson, K., Lim, N. K., Hoppe, H. J., and Reid, K. B. (1999) *Struct. Fold Des.* **7**, 255–264
28. Sheriff, S., Chang, C. Y., and Ezekowitz, R. A. (1994) *Nat. Struct. Biol.* **1**, 789–794
29. Weis, W. I., and Drickamer, K. (1994) *Structure* **2**, 1227–1240
30. Zhang, P., McAlinden, A., Li, S., Schumacher, T., Wang, H., Hu, S., Sandell, L., and Crouch, E. (2001) *J. Biol. Chem.* **276**, 19862–19870
31. Wallis, R., and Drickamer, K. (1997) *Biochem. J.* **325**, 391–400
32. Wallis, R., and Drickamer, K. (1999) *J. Biol. Chem.* **274**, 3580–3589
33. Brown-Augsburger, P., Hartshorn, K., Chang, D., Rust, K., Fliszar, C., Welgus, H. G., and Crouch, E. C. (1996) *J. Biol. Chem.* **271**, 13724–13730
34. Elhalwagi, B. M., Damodarasamy, M., and McCormack, F. X. (1997) *Biochemistry* **36**, 7018–7025
35. Bebbington, C. R., and Hentschel, C. C. G. (1987) in *DNA Cloning*, Vol. III (Glover, D. M., ed) pp. 163–188, IRL Press, Oxford
36. Fowler, W. E., and Erickson, H. P. (1979) *J. Mol. Biol.* **134**, 241–249
37. Olsen, D. R., Leigh, S. D., Chang, R., McMullin, H., Ong, W., Tai, E., Chisholm, G., Birk, D. E., Berg, R. A., Hitzeman, R. A., and Toman, P. D. (2001) *J. Biol. Chem.* **276**, 24038–24043
38. Schürmann, G., Haspel, J., Grumet, M., and Erickson, H. P. (2001) *Mol. Biol. Cell* **12**, 1765–1773
39. Erickson, H. P. (1974) *J. Supramol. Struct.* **2**, 393–411
40. Lu, C. L., Reedy, M., and Erickson, H. P. (2000) *J. Bacteriol.* **182**, 164–170
41. Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82**, 70–77
42. Aukhil, I., Joshi, P., Yan, Y., and Erickson, H. P. (1993) *J. Biol. Chem.* **268**, 2542–2553
43. Mosher, D. F., and Johnson, R. B. (1983) *J. Biol. Chem.* **258**, 6595–6601
44. Yee, V. C., Pratt, K. P., Côté, H. C. F., Le Trong, I., Chung, D. W., Davie, E. W., Stenkamp, R. E., and Teller, D. C. (1997) *Structure* **5**, 125–138
45. Kaires, N., Beisel, H. G., Fuentes-Prior, P., Tsuda, R., Muta, T., Iwanaga, S., Bode, W., Huber, R., and Kawabata, S. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 13519–13524
46. Haas, C., Voss, T., and Engel, J. (1991) *Eur. J. Biochem.* **197**, 799–803
47. Rothmann, A. B., Mortensen, H. D., Holmskov, U., and Hojrup, P. (1997) *Eur. J. Biochem.* **243**, 630–635