A switch in disulfide linkage during minicollagen assembly in *Hydra* nematocysts

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The smallest known collagens with only 14 Gly-X-Y repeats referred to as minicollagens are the main constituents of the capsule wall of nematocysts. These are explosive organelles found in *Hydra*, jellyfish, corals and other Cnidaria. Minicollagen-1 of *Hydra* recombinantly expressed in mammalian 293 cells contains disulfide bonds within its N- and C-terminal Cys-rich domains but no interchain cross-links. It is soluble and self-associates through non-covalent interactions to form 25-nm-long trimeric helical rod-like molecules. We have used a polyclonal antibody prepared against the recombinant protein to follow the maturation of minicollagens from soluble precursors present in the endoplasmic reticulum and post-Golgi vacuoles to the disulfide-linked insoluble assembly form of the wall. The switch from intra- to intermolecular disulfide bonds is associated with ‘hardening’ of the capsule wall and provides an explanation for its high tensile strength and elasticity. The process is comparable to disulfide reshuffling between the NC1 domains of collagen IV in mammalian basement membranes.

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Introduction

Nematocytes are highly specialized cells that serve for defense, capture of prey and locomotion in Cnidaria. These functions are mediated by a complex organelle, the nematocyst, of which a wide diversity of morphological types exists. A basic structure is, however, conserved: a capsule with a double-layered wall, an inverted tubule armed with spines and an operculum. Following appropriate stimulation, the capsule content is discharged leading to eversion of the tubule. The ultra-fast kinetics revealed by high-speed cinematography (Holstein and Tardent, 1984) show that during discharge an acceleration of $\geq 40\,000\text{ g}$ is generated. This specialized form of exocytosis is one of the fastest events in nature. It is driven

by the high internal pressure (150 bar) that results from the high concentration of poly- γ -glutamate and its bound cations (2 M) in the capsule matrix (Weber, 1990). The high internal pressure and the extraordinary speed of tubule evagination suggest the need for high tensile strength in the capsule and tubule wall.

Early experiments suggested that collagen-like molecules were constituents of the wall and tubule structures (Lenhoff *et al.*, 1957). These putative collagens were unusual in that they formed disulfide cross-linked polymers that are insoluble in SDS but easily soluble in the presence of a reducing agent (Blanquet and Lenhoff, 1966). A genetic screen for transcripts that are expressed in developing nematocytes identified a family of genes coding for Cys-rich collagen-related proteins and reconciled these earlier observations (Kurz *et al.*, 1991). As the Gly-X-Y stretches are the smallest known among collagen molecules, these proteins were termed minicollagens. Seven members have been cloned and all share a similar modular architecture (Kurz *et al.*, 1991, and C.N. David and T.W. Holstein, unpublished data). Furthermore, a related nematocyte-specific transcript has been identified in a reef-building coral (Wang *et al.*, 1995). The minicollagen-1 from *Hydra* encoded by the *N-COL-1* gene contains a central region of 14 Gly-X-Y triplets flanked at the N- and at the C-terminus by polyproline (or polyhydroxyproline) stretches of different lengths and by highly conserved Cys-rich regions (Figure 1). A model was proposed in which the polyproline helices and Cys-rich domains extend from the N- and C-termini of a central 12-nm-long collagen triple helix. Atomic force microscopy of mature nematocysts revealed that the capsule inner wall is made of bundles of collagen-like fibrils that were proposed to be disulfide cross-linked polymers of minicollagens (Holstein *et al.*, 1994). This structure was also shown to be surrounded by an outer wall of globular proteins of yet unknown primary structure, which are detected specifically by the monoclonal antibody H22 (mAB H22; Kurz *et al.*, 1991).

Biogenesis of the nematocyst capsule follows a very complex developmental pathway involving protein assembly. It leads to four different morphological types of nematocysts in *Hydra* [stenotele, desmoneme, holo- and atrichous isorhiza (Holstein *et al.*, 1990)]. Nematocysts self-assemble in a post-Golgi vacuole from materials supplied by vesicles from the endoplasmic reticulum (ER). Two layers, an electron-dense outer layer and an electron-lucent inner layer are gradually deposited on the inner side of the membrane of the post-Golgi vacuole to form the capsule wall. A cylindrical external tubule is then assembled at the apical end of the capsule. Upon completion, this tubule invaginates into the capsule and spines are assembled in the tubule lumen (Holstein, 1981). During the final maturation event the wall is hardened and

Fig. 1. Minicollagen-1 sequence and domain organization. Signal peptide (gray); propeptide (light blue); polypyrroline stretches (blue); Gly-X-Y, collagenous domain (red); Cys-rich regions with Cys residues indicated in green.

compacted to 50% of its initial thickness and the cyst attains its final shape. At the same time the capsule is filled with poly- γ -glutamate, giving rise to the high intracapsular pressure (Weber, 1990).

The assembly of minicollagens to the elastic and tense layers of the nematocyst wall is a highly interesting process, which may also serve as a model for the formation of collagenous layers in mammals. Collagens of invertebrates have recently attracted much interest because of their versatility (Engel, 1997), and minicollagen, which is the smallest known collagen so far, appeared to be one of the most interesting members. We have therefore recombinantly expressed one of the nematocyte minicollagens (minicollagen-1) in a eukaryotic expression system. The secreted recombinant minicollagen-1 was properly processed, had intrachain disulfide bonds and formed trimeric 25-nm-long rod-like structures. A polyclonal antibody prepared against recombinant minicollagen-1 recognized minicollagens in developing but not in mature nematocytes. During nematocyst formation the antiserum stained minicollagens in the capsule inner wall and in the tubule. During the process of wall hardening, a loss of staining was observed, which we interpret as a result of compaction of the structure. Biochemically this event was correlated with a switch from intrachain to interchain disulfide linkage, a process that is similar to the rearrangement of disulfide bonds between the NC1 domains of mammalian collagen IV during basement membrane formation (Siebold *et al.*, 1988).

Results

Secreted recombinant minicollagen-1 has intrachain disulfide bonds and assembles to a 25-nm-long trimeric rod-like structure

Minicollagen-1 was expressed in 293 cells using its native signal- and pro-sequences for secretion (Figures 1 and 2A). PCR was used to add a His₆ tag at the C-terminus, enabling purification of the minicollagen-1 by nickel-Sepharose chromatography (Figure 2A). Reducing SDS-PAGE (tricine buffer system) of conditioned medium from transfected cells revealed a 20 kDa product in the absence of ascorbic acid (Figure 2B, lane 1). In the presence of 50 and 200 μ g/ml ascorbic acid, an additional product at 21 kDa appeared in large quantities, suggesting hydroxylation as a post-translational modification (Figure 2B, lanes 2 and 3). The 20 and 21 kDa bands disappeared upon digestion with bacterial collagenase (data not shown).

Fig. 2. Minicollagen-1 expression and biochemical properties. (A) Design of minicollagen-1 and minicollagen-1-MBP fusions. (B) Recombinant expression. Conditioned medium of untransfected, minicollagen-1- and minicollagen-1-MBP-transfected cells was resolved by SDS-PAGE (Schägger and von Jagow, 1987) under reducing conditions. Mock, conditioned medium from untransfected cells. Lane 1, minicollagen-1 expression in the absence of ascorbic acid; lanes 2 and 3, minicollagen-1 expression in the presence of 50 and 200 μ g/ml ascorbic acid; lane 4, minicollagen-1-MBP expression in the absence of ascorbic acid; lanes 5 and 6, minicollagen-1-MBP expression in the presence of 50 and 200 μ g/ml ascorbic acid. (C) Effects of disulfide bonds on mobility of minicollagen-1. Equal amounts of non-reduced (lane 1) and reduced (lane 2) minicollagen-1 resolved by SDS-PAGE (Lämmli *et al.*, 1970). Proteins were visualized by silver staining.

N-terminal sequencing revealed that the mature minicollagen-1 began with the sequence DANP, indicating that the putative signal- and pro-sequences were removed during processing. Amino acid analysis of the 21 kDa minicollagen-1 product indicated that 14 of 58 Pro residues were hydroxylated. The predicted mass of the non-hydroxylated mature minicollagen-1 is 14 kDa. However, it is known that His-tagged proteins and collagens (Lillie *et al.*, 1987) migrate more slowly, and therefore have a higher apparent molecular weight in SDS-PAGE. Using a glycine buffer system (Lämmli, 1970), purified minicollagen-1 ran even more slowly at an apparent molecular weight of 26 kDa (Figure 2C, lane 2, see also Figure 4). Surprisingly, under non-reducing conditions minicollagen-1 ran at an apparent molecular weight of 24 kDa (Figure 2C, lane 1), suggesting a less expanded shape due to intrachain disulfide linkage.

Scanning transmission electron microscopy (STEM) of negatively stained minicollagen-1 in dilute acetic acid revealed rod-like particles with a tendency for lateral aggregation (Figure 3A). Prominent dark rods of 13 ± 1.3 nm were interlinked in some cases via thinner protrusions of 6–8 nm length (arrows in Figure 3A). In a collagen triple helix each Gly-X-Y repeat unit contributes a length of 0.86 nm (Traub and Piez, 1971) yielding a total length of 12 nm for the 14 Gly-X-Y repeats in the minicollagen-1, in close agreement with the length of the

Fig. 3. Electron micrographs of minicollagen-1 and minicollagen-1-MBP. (A) Scanning transmission electron micrographs of negatively stained minicollagen-1 in 0.1% acetic acid. Arrows indicate fine protrusions of 6–8 nm in length, which are enlarged in one case five times for better visibility (lower right panel). (B) Field of transmission electron micrographs of rotary-shadowed minicollagen-1 in 0.1% acetic acid. (C) Representative gallery of rotary-shadowed minicollagen-1-MBP molecules in 0.1% acetic acid as revealed by transmission electron microscopy. Bars: 50 nm.

observed rods. Polyproline or polyhydroxyproline are also known to be rod-like with a length of 0.31 nm per residue (Cowan and McGavin, 1955). The length of the longer 23 residue polyproline region (Figure 1) is therefore expected to be 7.1 nm, which matches the length of the protrusions. Minicollagen was also visualized by rotary shadowing. Isolated short rods were observed, which were frequently terminated by globular extensions (Figure 3B). The total length of the particles was 26 ± 2 nm, which has to be corrected for metal crystallite decoration by subtraction of 2.5 nm (Engel, 1994). The true length is therefore 24 ± 3 nm in agreement with the expected total length of the minicollagen-1 molecule. The diameter of the rods was similar to mammalian collagens observed by the same technique.

To confirm the trimeric nature of minicollagen-1, a fusion protein of the minicollagen-1 and maltose binding

Fig. 4. Western blot analysis of minicollagens in *Hydra* tissue and in purified capsules. Proteins were separated by SDS-PAGE (Lämmli *et al.*, 1970) and transferred to nitrocellulose membrane before immunodetection with minicollagen antibody. (A) Recombinant minicollagen-1 under non-reducing (lane 1) and reducing conditions (lane 2). (B) Chemical deglycosylation of minicollagens from mature capsules with anhydrous TFMS. Recombinant minicollagen-1 is shown as a control, before deglycosylation (lane 1) and after deglycosylation (lane 2). Untreated capsule material (lane 3) is compared with deglycosylated (lane 4) material. (C) Distribution of minicollagens in *Hydra* tissue. A flow chart depicts the sample preparation from animals cut into body column (b.) and head including tentacles (h.). Two body columns (lane 1) and two heads (lane 2) were completely solubilized in reducing sample buffer. For comparison 200 000 mature isolated capsules solubilized in reducing sample buffer are shown in lane 3. Ten *Hydra* heads and body columns were extracted with a buffer containing 2% CHAPS and 6 M urea but no reducing agent. The extractable protein was separated under non-reducing conditions (lane 4, body columns; lane 5, heads) or under reducing conditions (lane 6, body column; lane 7, heads).

protein (MBP) was expressed in 293 cells (see Figure 2A). MBP was chosen as the fusion partner because of its large globular form (42 kDa), lack of Cys residues and inability to aggregate in solution. The minicollagen-1-MBP fusion was efficiently secreted from 293 cells (Figure 2B, lanes 4–6) as a product of ~60 kDa, which fits well the predicted size of the fusion protein (58 kDa). At low ascorbic acid levels, yields decreased less than for minicollagen, but only the material expressed in the presence of 200 µg/ml ascorbic acid is expected to be fully hydroxylated and was used for further experiments. Transmission electron micrographs of rotary-shadowed minicollagen-1-MBP

revealed a bouquet-like arrangement of three globular domains (Figure 3C) clearly demonstrating a trimeric assembly imposed by the minicollagen triple helix.

Hydra minicollagens are produced as soluble precursors and are glycosylated

An antibody was generated by immunization of rabbits with recombinant minicollagen-1. The antiserum reacted strongly with minicollagen-1 in western blots (Figure 4A, lanes 1 and 2). As shown above, non-reduced and reduced recombinant minicollagen-1 ran at 24 and 26 kDa, respectively. A significantly lower antiserum reactivity was observed following reduction of minicollagen with β -mercaptoethanol (Figure 4A, compare lanes 1 and 2), suggesting that the epitopes recognized depend on the presence of intrachain disulfide bonds. Western blots of purified mature capsules that were solubilized with β -mercaptoethanol revealed two proteins at 34 and 40 kDa (Figure 4B, lane 3). Loss of these proteins upon digestion with bacterial collagenase indicated that the antiserum specifically recognizes collagen molecules (data not shown).

The difference in mass between single chains of recombinant minicollagen-1 and native capsule minicollagens led us to explore the possibility of the presence of post-translational modifications. We used TFMS (trifluoromethanesulfonic acid) to remove N- and O-linked glycosylations from β -mercaptoethanol-solubilized capsule minicollagens and observed that the 34 and 40 kDa bands were trimmed to a single 28 kDa band (Figure 4B, compare lanes 3 and 4), indicating ~15 and 30% glycosylation for each product. The deglycosylated product still had a slightly higher apparent molecular weight (28 kDa) than the recombinant deglycosylated minicollagen (26 kDa) under reducing conditions (Figure 4B, compare lanes 2 and 4). This difference could be the result of other post-translational modifications (see Discussion).

To distinguish between mature nematocysts and developing ones, the head including the tentacles and the body column were analyzed separately. The head contains only mature capsules, whereas the body column contains mainly differentiating nematocytes (David and Gierer, 1974). Figure 4C demonstrates that minicollagens are solubilized under reducing conditions from both the head and the body column (Figure 4C, lanes 1 and 2). Three major bands can be distinguished, of which the fastest and slowest approximately match the positions observed for minicollagens solubilized from isolated capsules (Figure 4C, lane 3). No minicollagen was extractable from head tissue when a reducing agent was not present in the extraction buffer (Figure 4C, lane 5). By comparison, minicollagen was extractable under non-reducing conditions from body column tissue (Figure 4C, lane 4). When run on a non-reducing gel, these soluble minicollagens ran as a smear between 30 and 34 kDa (Figure 4C, lane 4). When run on a reducing gel (Figure 4C, lane 6), however, they displayed a mobility similar to the minicollagens from mature capsules (compare with Figure 4C, lane 3).

These data indicate the presence of a soluble, monomeric form of minicollagen in body column tissue where capsules are forming, and of a polymeric, disulfide-linked form of minicollagen in mature capsules.

Fig. 5. Immunolocalization of minicollagen in a whole mount labeled with minicollagen antibody (red) and mAb H22 (green). A fully developed bud is shown using confocal microscopy. Bar: 100 μ m.

Minicollagens are localized in the wall of developing nematocysts

To localize minicollagens during nematocyst morphogenesis, whole mounts of *Hydra* double stained with the minicollagen antibody and the mAb H22 were examined with a confocal laser microscope. The mAb H22 reacts with the outer wall of nematocysts at all developmental stages (Kurz *et al.*, 1991). Figure 5 shows the distribution of nematocysts in a fully developed bud stained with both antibodies. H22 mAb (green) stained both immature and mature nematocysts throughout the animal. In contrast, the minicollagen antibody (red) stained only clusters of cells with developing capsules in the gastric region. Minicollagen antibody reactivity is thus restricted to immature morphogenetic stages that are found throughout the ectoderm of the gastric region in clusters of 8–32 cells (David and Challoner, 1974). The lack of minicollagen antibody staining in the head region is consistent with the absence of differentiating nematocytes in this tissue.

The distribution of both antigens (minicollagen, red; H22, green) was examined at higher resolution in developing nematocyst capsules and correlated with electron micrographs of thin sections. A schematic diagram in Figure 9 summarizes the distribution of both antigens in different morphogenetic stages. Nematocysts differentiate in post-Golgi vacuoles, to which material is constantly added by vesicle fusion (Figure 6C; Holstein, 1981). This structure is surrounded by an elaborate ER and several Golgi cisternae at the apical end of the growing capsule. The confocal image of a nest of nematocytes at a very early stage of development reveals minicollagen staining throughout the cell except for the nucleus, indicating the presence of minicollagen in the ER and Golgi (Figure 6A and B). In the developing capsule, the H22 antigen clearly forms the outer layer of the cyst adjacent to the vacuole membrane (Figure 6A), whereas minicollagen staining seems to be confined to the capsule matrix (Figure 6B).

At a later stage of development, when the inner and outer wall are more clearly formed, an optical section of a capsule (Figure 7A) reveals that the minicollagen layer

Fig. 6. Early stages of nematocyst morphogenesis. (A–B) Nest of developing nematocytes in the gastric region viewed using confocal microscopy. (A) Optical section of a nest of nematocytes double labeled with minicollagen antibody (red) and mAb H22 (green). The cyst (arrow) is already clearly distinguishable by mAb H22 staining in the wall. (B) Same optical section showing minicollagen staining only. Note the presence of minicollagen in the ER and the capsule (arrow). (C) Electron micrograph of a single nematocyte with growing capsule at a similar stage (iw, inner wall; N, nucleus; ow, outer wall). Bars: (A and B) 5 μ m; (C) 1 μ m.

Fig. 7. Developing nematocytes before and during formation of the external tubule. Nematocytes in the gastric region viewed using confocal (A–D) and electron (E) microscopy. (A) Optical section of a nematocyst (isorhiza) double labeled with mAb H22 (green) and minicollagen antibody (red). H22 is detected in the outer wall, minicollagen is detected in the inner wall and to a lesser extent in the matrix. (B) Projection view of a nematocyst (stenotele) labeled with minicollagen antibody (red). At the apex of the cyst (arrow), minicollagen-filled vesicular structures are observed. (C) Three-dimensional reconstruction of the capsule shown in (B) in simulated fluorescence process mode. (D) Optical section of a nematocyst (desmoneme) with wall and external tubule labeled with minicollagen antibody (red). Vesicles fuse at the tip of the tubule (arrows). (E) Electron micrograph of a nematocyst (desmoneme or isorhiza) forming its external tubule (et, external tubule; iw, inner wall; ow, outer wall). Bars: (A, B and D) 2 μ m; (E) 1 μ m.

corresponds to the electron-lucent inner wall observed in electron micrographs (Figures 6C, 7E and 8D). This layer seems to be capable of extensive rearrangement at this stage, since both confocal and electron microscope images show local thickenings of the inner wall (Figure 7A and E), which are absent at later stages. In the matrix, minicollagen staining is rather diffuse but also exhibits bright dots (Figure 7A) corresponding to the granular or aggregated material that is also observed in electron micrographs (Figure 7E). The same morphogenetic stage is shown in Figure 7B and C, and reveals how vesicular structures fuse continuously at the apex of the cyst to provide material for the capsule. Before the cyst has attained its final size, it forms a tubule at one end that grows by vesicle addition. This morphogenetic stage is illustrated in Figure 7D

(confocal optical section) and E [electron microscopy (EM)]. Whereas the cyst is stained brightly, tubule staining is much weaker (Figure 7D). This is consistent with EM images that show the tubule wall to be continuous with the cyst inner wall but with a much thinner electron-lucent layer (Holstein, 1981). Minicollagen-filled *trans*-Golgi network is also observed at the tip of the growing tubule.

After vesicle addition has stopped and the tubule has inverted into the cyst the nematocyst undergoes a maturation process that includes compaction of the inner wall and hardening of the whole structure (Holstein, 1981; Watson and Mariscal, 1984). At the same time, the capsule is filled with osmotically active poly- γ -glutamate giving rise to the high intracapsular pressure. In a cluster of developing nematocysts undergoing compaction, different

Fig. 8. Nematocysts undergoing wall maturation. Nematocyst in the gastric region viewed using confocal (A–C) and electron microscopy (D). (A) Projection view of a nematocyst nest (stenoteles) triple labeled with minicollagen antibody (red), mAb H22 (green) and DAPI (blue). The H22 antibody stains all capsules in the nest. Only the irregularly shaped capsules that have not yet undergone maturation are stained with the minicollagen antibody. (B) Minicollagen staining in a single optical section through the same nest. (C) Minicollagen staining in a nematocyst nest (stenoteles) in which capsules are undergoing compaction (projection view). Capsules with regular shape and weak minicollagen antibody staining are marked by arrows. (D) Electron micrograph of a nematocyst (stenotele) with invaginated tubule (iw, inner wall; ow, outer wall; s, stylets; t, invaginated tubule). This nematocyst is almost mature, and represents the same stages as the ones marked by arrows in (C). Bars: (A–C) 5 μ m; (D) 2 μ m.

Fig. 9. Schematic drawing of minicollagen and H22 antigen distribution during nematocyst morphogenesis. The stages of morphogenesis are outlined for a stenotele but also apply to a desmoneme or an isorhiza. Minicollagen is shown in red, the outer wall antigen H22 in green and membranes are drawn as blue lines. (A) A newly developing nematocyst grows in a post-Golgi vacuole surrounded by an extensive ER network and several Golgis. Minicollagen is synthesized in the ER and gets sorted via the Golgi to the cyst vesicle. (B) After accumulation inside the growing cyst, minicollagen is deposited on the pre-existing outer wall to form the inner wall layer (only one of several Golgis is shown). (C) By fusion of minicollagen-filled vesicles at one end of the capsule a long external tubule starts to grow and elongates, while the cyst itself still grows in diameter. (D) After the cyst has reached its final size, the tubule invaginates into the cyst (not shown), and is no longer stained with the minicollagen-1 antibody (tubule now shown as gray structure). Spines are then formed in the lumen of the inverted tubule (Koch *et al.*, 1998). (E) During the final step of maturation, a gradual loss of minicollagen antibody staining (now shown as red thin line) reflects the wall compaction and hardening process. c, capsule; G, Golgi; N, nucleus; rER, rough endoplasmic reticulum; s, stylets; t, tubule.

intermediate stages can be observed (Figure 8A, B and C). Nematocysts that have not yet compacted display brighter minicollagen staining than at any other morphogenetic stage and exhibit an irregular shape due to deformation

during fixation. Nematocysts that have completed compaction exhibit no or highly reduced minicollagen staining and have the smooth form of mature capsules. These capsules are no longer susceptible to deformation through

fixation, and hence we define this stage as having a 'hardened' wall (Figure 8D). Mature capsules showed no reaction with minicollagen antibody (see Figure 5) except for a very weak staining after post-fixation treatment with β -mercaptoethanol. Treatment with pronase after fixation to facilitate the penetration of the antibody did not increase staining (data not shown). We interpret the loss of antigen reactivity as the result of compaction of the inner wall due to minicollagen polymerization and disulfide linkage rearrangement (see Discussion).

Discussion

The assembly of minicollagens into the nematocyst wall is a complex process involving vesicular transport of a soluble form and tight association by disulfide rearrangement. Although we have analyzed the properties only of minicollagen-1 in this study, we envisage that our results apply to other known minicollagens with similar domain architecture. The assembly process serves as a model for the not yet well understood assembly of basement membranes from collagen IV in *Drosophila*, *Caenorhabditis elegans* and mammals (Yurchenco and O'Rear, 1994) in which disulfide isomerization in the Cys-rich NC1 domains is also an essential step (Siebold *et al.*, 1988).

Biochemical and structural properties of minicollagen-1

In order to obtain insight into the biochemical and structural properties of minicollagens, we used a eukaryotic expression system to recombinantly produce the minicollagen-1 protein. In this system, the native signal- and pro-sequences that are used for sorting the protein to the post-Golgi vacuole in which the nematocyst forms (Anderluh *et al.*, 2000) were removed and enabled secretion of the protein into the medium. Instead of forming a disulfide-linked polymer as observed in mature nematocysts, minicollagen-1 assembled into 25-nm-long trimeric rods that displayed intrachain disulfide bonds and were soluble.

The structural characteristics of the expressed minicollagen molecule fit remarkably well with those predicted from its sequence using the known structures of collagen and polyproline II helices (Kurz *et al.*, 1991; see also Results). Minicollagen-1 was efficiently hydroxylated in the presence of ascorbic acid (Figure 2B). Since mammalian cells only express a prolyl-4-hydroxylase, capable of hydroxylating the Y position in a Gly-X-Y triplet (Bateman *et al.*, 1996), the observation of 14 hydroxyproline residues in minicollagen suggests that all of the Y positions of the Gly-X-Y domain are hydroxylated. Hydroxyproline in the Y position is required for triple helix stabilization (Traub and Piez, 1971). Sequencing of peptides of native *Hydra* minicollagen-1 revealed that most of the prolines in the polyproline part were also hydroxylated (R.Deutmann, personal communication). The higher degree of proline hydroxylation in *Hydra* minicollagen compared with the recombinant minicollagen-1 could also be the reason for its higher apparent molecular weight in SDS gels (Figure 4B), since hydroxylation of proline alters the electrophoretic mobility (Phelps and Floros, 1988). Hydroxylation of polyproline

has also been shown to occur in extracellular matrix proteins of higher plants (Kieliszewski and Lamport, 1994) and the colonial algae *Volvox* (Ertl *et al.*, 1989).

Like most collagens, minicollagen-1 exhibited limited solubility in dilute acidic acid, and monomers were detectable by rotary shadowing (Figure 3B). Even under these acidic conditions STEM revealed that minicollagen rods exhibit the potential of interactions by their ends and lateral aggregation to superstructures (Figure 3A). When the buffer was switched to neutral conditions, hydroxylated (but not non-hydroxylated) minicollagen aggregated and could be pelleted by minifuge centrifugation (data not shown). Such behavior is characteristic of many collagens that can self-assemble *in vitro* into ordered fibrillar structures at neutral pH. This fibril-forming ability is known to be encoded in the structure of the collagen triple helix (Bateman *et al.*, 1996) and therefore is dependent on proper proline hydroxylation. We were unable to observe higher order structures by EM, since at neutral pH the minicollagen formed highly viscous aggregates that did not permit EM sample preparation.

Soluble precursor minicollagens assemble to a superstructure through non-covalent interactions during nematocyst development

The observation that 293 cells produce triple helical minicollagen molecules with intrachain disulfide bonds, instead of a disulfide-linked polymer, led us to hypothesize the existence of a soluble minicollagen precursor in developing capsules. Using the minicollagen antibody, we were able to identify such a soluble precursor and to show that it was only present in tissue containing developing nematocysts (Figure 4C). This precursor displayed the same apparent molecular weight as the minicollagen from isolated capsules, indicating that it was glycosylated and probably had left the ER. The increased mobility of the precursor in non-reducing gels (Figure 4C, lane 4) suggests the presence of intrachain disulfide bonds. Thus, our results support the idea that minicollagens are not gradually incorporated into a disulfide-linked polymer during formation of the capsule wall. Rather, minicollagen trimeric units appear to self-assemble through non-covalent interactions into an ordered superstructure, which is visible in electron micrographs as a thick electron-lucent layer. Such a superstructure was suggested for minicollagen at a neutral pH, and, as for classic collagens, should be encoded in the collagen triple helical domain.

Minicollagens appear to store their disulfide bonds in an intrachain linkage to avoid cross-linking accidents that would result from a high local concentration of Cys residues in the oxidative environment of the post-Golgi vacuole. We propose two possible arrangements of the intrachain disulfide bonds in minicollagen-1 in Figure 10A. These combinations are based on a comparison of proteins with known disulfide patterns (EGF, laminin EGF domain, coagulagen, hirustasin, NC1 domain of collagen IV, collagen III, EGF receptor, cartilage matrix protein, cartilage oligomeric matrix protein precursor). Linkage of Cys-Cys, which seems sterically impossible, as well as the linkage of CysXXXCys were not observed in these proteins. Excluding these linkages, there are three remaining possibilities for the arrangement of disulfides within

the Cys-rich domain of minicollagen-1, of which the two sterically more likely are shown.

Our results show that the minicollagens have a strong tendency to self-assemble into larger aggregates (Figure 3A). Under *in vivo* conditions, however, additional positional cues may be present inside the post-Golgi vacuole to organize the minicollagens into an ordered wall structure. Immunofluorescence and EM pictures of early stages of cyst development show that minicollagens are always deposited on a pre-existing outer wall consisting of the H22 antigen (Figures 6 and 7). This structure could provide nucleation sites on which minicollagens assemble to form the inner wall. The nature of the outer wall and the molecules that provide such positional cues remain to be investigated.

Isomerization of intra- to interchain disulfide bonds accompanies polymerization of minicollagens to the mature capsule wall

The presence of soluble precursor minicollagen in developing capsules and polymeric disulfide-linked minicollagen in mature capsules implies the existence of a mechanism converting one configuration to the other. This mechanism appears to be associated with 'wall hardening', a process that results in a 50% compaction of the inner wall (Holstein, 1981; Watson and Mariscal, 1984). During this process we observed a dramatic loss of minicollagen antibody staining (Figure 8) due to masking of minicollagen epitopes. Since few nests exhibit this feature, we estimate that compaction is a relatively fast process, occurring within 1–2 h. Just before wall hardening, minicollagen antibody staining is at its highest intensity, suggesting a high accessibility of the antibodies to the minicollagens. These observations suggest a close link between a conformational change in the inner wall and disulfide isomerization. Our concept of polymerization by formation of cross-links is supported by earlier experiments in which nematocysts of a sea anemone were studied by EM (Watson and Mariscal, 1984). Treatment of animals with performic acid, which oxidizes disulfides to sulfonates, and therefore opens the disulfide bonds, induced a thickening of the inner wall of mature nematocysts to the dimensions that were observed in late stage immature cysts. This observation supports the idea that thinning of the capsule wall during cyst formation results from the formation of intermolecular disulfide cross-links.

Disulfide isomerization during the extracellular polymerization of mammalian collagen IV by dimerization of its C-terminal non-collagenous (NC1) domains was investigated in molecular detail (Siebold *et al.*, 1988). It

was found that intrachain disulfides in NC1 monomers open and recombine to form disulfide bonds between two different molecules. Interestingly, the same Cys residues are involved in intrachain and interchain bonding (Siebold

Fig. 10. Structural model for minicollagen assembly. (A) Intrachain linkage in the soluble precursor. Two alternative arrangements of the intramolecular disulfide bonds within the N-terminal Cys-rich domain of minicollagen-1 are shown. (B) Model of assembly. Minicollagens are synthesized as soluble precursors that have an intrachain disulfide linkage. During the final maturation step, disulfide isomerization from intra- to intermolecular disulfide linkage leads to minicollagen polymerization. Polymeric minicollagen fibers stretch elastically to store spring-like forces that are released during explosive exocytosis (see Discussion). For simplicity, the conformational change of only one disulfide bridge per Cys-rich assembly region is shown.

Electron microscopy

STEM was performed with a Vacuum Generators HB-5 scanning transmission electron microscope controlled by a modular computer system (Tietz, Video and Image Processing Systems, D-8035 Gaunting). The solution of minicollagen-1 in 0.1% acetic acid was adsorbed to freshly glow-discharged thin carbon films supported by thick perforated carbon layers and negatively stained with uranyl formate following standard procedures (Engel, 1994). For rotary shadowing, minicollagen-1 or minicollagen-1-MBP was sprayed onto freshly cleaved silica, shadowed at an angle of 9° and visualized after carbon coating and replication (Engel, 1994) by a Phillips 400 transmission electron microscope. Electron micrographs of *Hydra* ultra-thin sections were performed as described in Holstein (1981).

Strains and culture conditions

Hydra magnipapillata strain 105 was used for all experiments. Animals were cultured in M solution at 18°C (Loomis and Lenhoff, 1956) and fed every day with *Artemia nauplii*. Animals were starved for 24 h before experiments.

Minicollagen antibody

A polyclonal antibody directed against minicollagen-1 was generated in rabbits by Eurogentec Bel S.A. (4102 Ougree, Belgium). Purified minicollagen-1 in 20 mM Tris-HCl pH 7.4 containing 2 M urea was injected into rabbits. Immunization was carried out following a standard protocol using 100 µg of the antigen per injection.

Electrophoresis and western blot analysis

Proteins were solubilized in sample buffer (2% SDS, 0.0625 Tris-HCl pH 6.8, 10% glycerol) and resolved by SDS-PAGE on 12% gels using either the tricine buffer system according to Schägger and von Jagow (1987) or the glycine buffer system according to Lämmli (1970). As the two systems resulted in slightly different apparent molecular weights, the system used is indicated in the figure legend. To visualize proteins, the gels were silver stained.

Capsules were isolated as described (Weber, 1990; Kurz *et al.*, 1991) and were solubilized by heating (30 min, 60°C) in sample buffer supplemented with 0.8 M β-mercaptoethanol. For analysis of *Hydra* extracts, *Hydra* without buds were decapitated below the tentacles. Heads (including tentacles) and body columns were frozen for 1 h to break up the tissue and then extracted in a buffer containing 6 M urea and 2% 3-(3-choloamidopropyl)diethylammonio-1-propanesulfonate (CHAPS). Insoluble material was pelleted and the supernatant was supplemented with sample buffer with or without 0.8 M β-mercaptoethanol. For chemical deglycosylation, 2 million capsules of recombinant minicollagen-1 were solubilized in lysis buffer and subsequently precipitated in trichloroacetic acid (TCA) together with fetuin (200 µg/reaction) as a carrier glycoprotein. Chemical deglycosylation was performed as described by Edge *et al.* (1981) by incubation of the dried protein pellet in a 2:1 mixture of anhydrous TFMS:anisole for 2.5 h on ice. After a further incubation of 2 h at -20°C the TFMS was removed by TCA precipitation. The samples were then solubilized in sample buffer containing 0.8 M β-mercaptoethanol. All protein extracts were separated by SDS-PAGE and transferred to a nitrocellulose membrane as described by Towbin *et al.* (1979). Minicollagen antibody was used at dilutions between 1:500 and 1:1000. The primary antibody was detected using an anti-rabbit antibody coupled to horseradish peroxidase (1:10 000) and the ECL chemoluminescence system (Amersham).

Recombinantly prepared and extracted proteins were assayed by SDS-PAGE after incubation with 10 U/ml bacterial collagenase from *Clostridium histolyticum* with 180 U/mg activity and low other proteolytic activities (Worthington, Code CLS3) in 50 mM Tris buffer pH 7.4 at 37°C for 4 h. In controls actin was undigested under the same conditions.

Immunofluorescence

Animals were relaxed in 2% urethane in M solution for 2 min and fixed either in Lavdovsky's fixative (50% ethanol, 3.7% formaldehyde, 4% acetic acid in water) or in 4% formaldehyde or paraformaldehyde for 24 h. The fixative was removed by several washes in phosphate-buffered saline (PBS), and membranes were opened by incubation for 30 min in 0.1% Triton X-100. For double staining, minicollagen-1 antibody was diluted 1:500 in mAb H22 (Kurz *et al.*, 1991) and incubated with animals overnight. After several washes in PBS, the animals were incubated for 5 h in anti-rabbit antibody coupled to ALEXA-568 fluorochrome and anti-mouse antibody coupled to ALEXA-488 fluorochrome (Molecular Probes), both diluted 1:400 in PBS with 1% bovine serum albumin. The

animals were then washed again several times in PBS and nuclei were stained with 0.5 µg/ml of the DNA-specific stain 4',6-diamidino-2-phenylindole dichloride (DAPI). Finally, animals were mounted permanently in ProLong mounting media with antifade (Molecular Probes).

Whole mounts were viewed and documented on a confocal laser scanning microscope (Leica TCS SP). Single photon excitation was generated with an argon-krypton laser and 2-photon excitation with a femtosecond pulsed Ti:sapphire laser (Tsunami, Spectra Physics) pumped by a Nd:YVO₄ laser (Millenia V, Spectra Physics). The 2-photon laser was used for excitation of DAPI. The micrographs are shown either as single optical sections or as projections through a series of optical planes (indicated in the legend). Overlays of multiple channels and projections were carried out using the Leica TCS software 1.6 and Leica confocal software 2.00. The latter was also used for the 3D reconstruction in simulated fluorescence process (SFP).

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