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Invited Review

Enzymology of the nematode cuticle: A potential drug target?

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ABSTRACT

All nematodes possess an external structure known as the cuticle, which is crucial for their development and survival. This structure is composed primarily of collagen, which is secreted from the underlying hypodermal cells. Extensive studies using the free-living nematode *Caenorhabditis elegans* demonstrate that formation of the cuticle requires the activity of an extensive range of enzymes. Enzymes are required both pre-secretion, for synthesis of component proteins such as collagen, and post-secretion, for removal of the previous developmental stage cuticle, in a process known as moulting or exsheathment. The excretion/secretion products of numerous parasitic nematodes contain metallo-, serine and cysteine proteases, and these proteases are conserved across the nematode phylum and many are involved in the moulting/exsheathment process. This review highlights the enzymes required for cuticle formation, with a focus on the post-secretion moulting events. Where orthologues of the *C. elegans* enzymes have been identified in parasitic nematodes these may represent novel candidate targets for future drug/vaccine development.

1. The cuticle

All nematodes are encased in an exoskeleton (known as the cuticle), a structure key to the success and diversity of nematode species. This complex extracellular matrix covers the outermost layer of cells and is required for body shape, movement, and functions as the primary interface with the environment (Fig. 1A). The cuticle structure and its biogenesis have been most extensively studied in the free-living model nematode *Caenorhabditis elegans* (Singh and Sulston, 1978; Page and Winter, 2003; Page and Johnstone, 2007). As a new cuticle is generated for each developmental stage, it is synthesised five times during the nematode lifecycle, with synthesis of the first cuticle beginning during late embryogenesis. Progression through development requires that the cuticle from the previous stage is shed and replaced with the new cuticle in a process known as moulting. The importance of the cuticle in maintaining body shape has been illustrated by genetic analysis in *C. elegans*. Strains carrying mutations in either the structural components of the cuticle, or the enzymes required for cuticle formation, result in drastically altered body morphology or lethality (Kramer, 1997; Page and Winter, 2003; Page and Johnstone, 2007).

Collagen and collagen-like proteins, at 80% of total protein, constitute the vast majority of the cuticular structural components. Compared with vertebrate collagen monomers, which are large and consist of long uninterrupted runs of the defining Gly-X-Y motif (where Gly is glycine, and X and Y are most often proline and hydroxyproline, respectively), nematode collagen monomers are generally smaller, around 35 kDa, and contain multiple interruptions within the Gly-X-Y repeat regions. The *C. elegans* cuticle collagen family consists of 167 members, 22 of which result in informative body morphology defects when mutated; these include phenotypes known in *C. elegans* nomenclature as dumpy (or Dpy, which are short and fat), roller (or Rol, helically twisted), long (or Lon), squat (or Sqt, short and twisted) and blistered (or Bli, fluid-filled blistering of the cuticle) (Page and Johnstone, 2007). Similar morphological phenotypes are found in mutants where the enzymes required for collagen and cuticle synthesis are defective (Page and Winter, 2003; Page and Johnstone, 2007). In addition to collagen, an unusual highly cross-linked class of insoluble protein called cuticlin is present in the nematode cuticle (Sapio et al., 2005), with *C. elegans* cuticlin mutants displaying dumpy morphological defects in specific developmental stages (Muriel et al., 2003; Sapio et al., 2005).

Collagen biogenesis is a complex, multi-step process with modifications that occur both intra- and extra-cellularly and requires the function of numerous enzymes (Fig. 1B). Some of the key enzymes involved in this pathway in *C. elegans* will be discussed in detail and their relevance to important human and animal parasitic nematodes will be highlighted.

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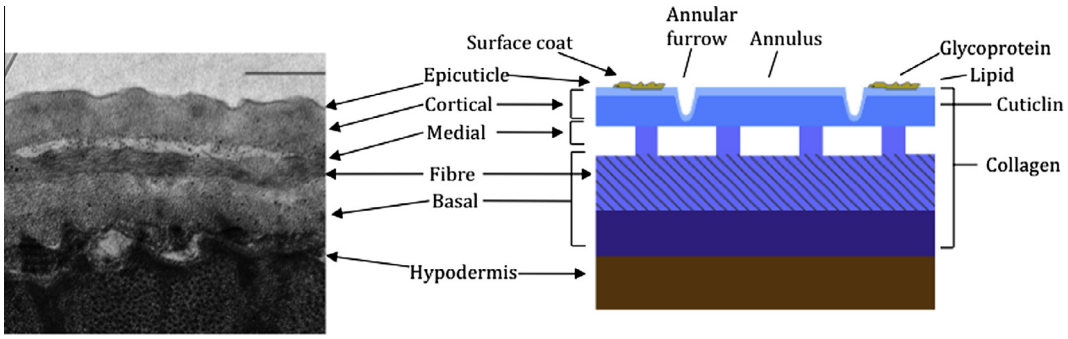

Fig. 1A. The organization and structure of the *C. elegans* cuticle. Left image is a transmission electron micrograph (TEM) depicting a longitudinal cross-section of the adult cuticle. Right panel is a cartoon depiction highlighting the distinct structural layers and their composition. With the exception of the epicuticle and surface coat, collagens are present in all major layers. Cuticlins are restricted to the cortical layer. The epicuticle contains lipids and is covered by a glycoprotein-rich coat (scale bar 1 μm).

2. Enzymatic modifications occurring within the endoplasmic reticulum

2.1. Hydroxylation of proline residues

The first step in collagen synthesis involves the co-translational hydroxylation of Y position proline residues to 4-hydroxyproline in the Gly-X-Y domain, a modification that is required to stabilise the final assembled collagen triple helix. This reaction is catalysed by collagen prolyl 4-hydroxylase (C-P4H), which in multi-cellular animals are oligomeric enzymes consisting of α subunits, which contain the active site residues, and β subunits, formed by protein disulphide isomerase (PDI). Our lab has studied C-P4H extensively in the free-living nematode *C. elegans* where they are developmentally essential. *C. elegans* lacking a single C-P4H α subunit (DPY-18) are viable but show abnormal body morphology, cuticle structure, collagen localisation, and reduced levels of cuticular 4-hydroxyproline (Winter and Page, 2000), while combined loss of both α subunits (DPY-18 and PHY-2) results in embryonic lethality (Winter and Page, 2000; Winter et al., 2007b). Oligomeric C-P4Hs in all species examined contain only one type of β subunit PDI, which is present in all forms of the complex. Therefore, in *C. elegans* single loss of the β subunit (PDI-2) results in phenotypes equivalent to combined disruption of both α subunits (Winter and Page, 2000; Winter et al., 2007b) (Fig. 2). Recombinant *C. elegans* C-P4H are effectively inhibited using co-substrate analogues and *in vivo* these compounds replicate the phenotypes found by genetic disruption in *C. elegans* (Myllyharju et al., 2002). *In vitro* and *in vivo* work demonstrated that the *C. elegans* C-P4H complexes were formed from combinations of subunits that are unique to nematodes (Myllyharju et al., 2002). Similar analysis of a close relative of *C. elegans*, *Caenorhabditis briggsae*, revealed further nematode-specific C-P4H complexes that differ significantly from their vertebrate counterparts (Winter et al., 2007b).

Using a novel RNAi approach, our lab demonstrated that C-P4H is also developmentally essential in the human infective filarial nematode *Brugia malayi* (Winter et al., 2013). Analysis of the *B. malayi* genome (Ghedin et al., 2007) aided identification of all potential C-P4H subunits. Simultaneous RNAi of both α subunits (*Bma*-PHY-1 and -2) in cultured *B. malayi* adult females resulted in a highly penetrant body morphology defect in the released microfilariae (L1s) (Winter et al., 2013) (Fig. 2). This effect was replicated following RNAi of the single β subunit (*Bma*-PDI-2) (Winter et al., 2013). These results are supported by previous studies which showed that 4-hydroxyproline levels in *B. malayi* cuticle collagens were similar to those of *C. elegans* (Cox et al., 1981; Petralanda and Piessens, 1991) and that moulting of cultured *B. malayi* L3s was dependent on addition of ascorbate, a co-factor essential for C-P4H activity (Rajan et al., 2003). While the *B. malayi* RNAi results clearly mirrored the *C. elegans* genetic analysis, biochemical approaches revealed unusual aspects of the parasitic nematode proteins. In contrast to α subunits from every other multi-cellular species examined, recombinant *Bma*-PHY-1 and -2 proteins did not require PDI for solubility (Winter et al., 2003). Also, co-expression of all three *B. malayi* C-P4H components (*Bma*-PHY-1, -2 and *Bma*-PDI-2) in all combinations failed to produce highly active enzyme. In addition, the *B. malayi* proteins did not complement a *C. elegans* C-P4H mutant. Importantly, lack of complementation using *B. malayi* proteins is not a general phenomenon, as *Bma*-PDI-2 can complement a *C. elegans pdi-2* mutant. This also confirmed that *Bma*-PDI-2 was a *bona fide* C-P4H β subunit. Further investigation into the *B. malayi* proteins revealed that parasite-derived, but not recombinant, *B. malayi* material contained a non-reducible covalent bond linking the subunits. This indicated that *B. malayi* C-P4H activity was dependent on this modification and its absence in recombinant proteins accounted for lack of activity/function in the assays employed. Enzymes from additional parasitic nematode species will need to be examined to determine if the *B. malayi* enzymes are unique in this respect. However, a covalent crosslink is not found between C-P4H subunits from any other species examined to date, including the extensively studied vertebrate enzymes. It is possible that the major differences in enzyme assembly between nematodes and vertebrate enzymes may be exploitable in the development of nematode-specific C-P4H inhibitors.

2.2. Procollagen registration: disulphide bond formation

The next important step in collagen biogenesis is the registration and trimerisation of the collagen chains. Registration involves the correct association of the monomers and is catalysed by PDI through disulphide bond formation of highly conserved C-terminal cysteine clusters in the cuticle collagens. This event may be key to the selection and assembly of the correct partners to form homo- or indeed hetero-trimers. In addition to the role in proline hydroxylation, it has been established that PDI-2 is also involved in this oxidative registration step in *C. elegans* (Winter et al., 2007b). Mutations in *pdi-2* cause severe cuticle defects and adult sterility, whereas the combined mutations of *pdi-1*, *pdi-2* and *pdi-3* cause embryonic lethality and it was concluded that PDI-2 performs two essential functions during morphogenesis: one that is C-P4H dependent and the other contributing to disulphide bond formation. The importance of disulphide bonds and the oxidative/reductive state of the cuticle has also been found to be critical during the moulting process (Stenvall et al., 2011) and will be addressed further under the moulting section.