Ultrastructure of the proteoliaisin-ovoperoxidase complex and its spatial organization within the Strongylocentrotus purpuratus fertilization envelope

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SUMMARY

Ovoperoxidase is a cortical granule-derived enzyme that hardens the sea urchin fertilization envelope by catalyzing the formation of dityrosine residues. Ovoperoxidase works in concert with a second protein, proteoliaisin, which anchors ovoperoxidase to the nascent fertilization envelope in a divalent cation-dependent manner. In this study, we examined the Ca²⁺-dependent interaction of proteoliaisin with ovoperoxidase in rotary-shadowed Pt replicas. Ovoperoxidase, a uniformly sized globular molecule, binds to a distal portion of rod-shaped proteoliaisin when low concentrations of Ca²⁺ are present. Higher Ca²⁺ concentrations lead to the formation of extended proteoliaisin strands that are decorated along their lengths with ovoperoxidase. Using immunogold labeling, we also examined the assimilation of these two proteins into the fertilization envelope in quick-frozen, deeply etched samples. Both proteins are abundant in the fertilization envelope as early as one minute after fertilization. Coincident with paracrystalline coating of the envelope, the labeling density is markedly reduced, suggesting that antigenic sites may be masked by the paracrystalline coat. This suggests that the ovoperoxidase-proteoliaisin complex resides within the central portion of the fertilization envelope, rather than in the paracrystalline coat.

Key words: fertilization envelope, proteoliaisin, ovoperoxidase, sea urchin, egg envelope

INTRODUCTION

The sea urchin fertilization envelope (FE) is a remarkably stable macromolecular assembly, which acts as a permanent block to polyspermy and shelters the embryo during early development. The FE self-assembles in seawater when cortical granule contents interact with the vitelline layer, a glycoproteinaceous layer surrounding unfertilized eggs (Glabe and Vacquier, 1978; Kinsey and Lennarz, 1981; Rossignol et al., 1984). The first step occurs when cortical granule proteases cleave vitelline layer/plasma membrane linkages allowing the vitelline layer to elevate off the egg surface (Vacquier et al., 1972; Longo and Schuel, 1973; Carroll and Epel, 1975). Next, cortical granule proteins modify and assimilate into the vitelline scaffold, culminating with the deposition of a paracrystalline layer on either side of the assembling structure (Chandler and Heuser, 1980; Chandler and Kazilek, 1986). During the early stages of assembly, the nascent FE is referred to as a ‘soft’ structure, since it can be readily dissolved by reducing agents or in divalent cation-depleted solutions (Paul and Epel, 1971; Veron et al., 1977; Kay et al., 1982). In contrast, the ‘hardened’ FE is remarkably resistant to disruption by chemical, mechanical and enzymatic treatments, as a consequence of covalent cross-linking (reviewed by Kay and Shapiro, 1985).

Ovoperoxidase is the enzyme responsible for hardening the FE by catalyzing the formation of dityrosine residues (Foerder and Shapiro, 1977; Hall, 1978). Ovoperoxidase uses H₂O₂ produced in a CN⁻-insensitive respiratory burst at fertilization (Foerder et al., 1978), as an oxidative substrate to form crosslinks between appropriately positioned tyrosine residues (Foerder and Shapiro, 1977; Hall, 1978; Kay et al., 1982). Ovoperoxidase does not bind directly to the nascent FE, instead it is anchored via a divalent cation-dependent interaction with an intermediary protein, proteoliaisin (Weidman et al., 1985). Proteoliaisin is a 230 kDa, highly asymmetric protein that colocalizes with ovoperoxidase in cortical granules (Weidman et al., 1987; Somers et al., 1989). Proteoliaisin binds to the vitelline layer of unfertilized eggs, and presumably the nascent FE, in a divalent cation-dependent manner that is independent of its association with ovoperoxidase (Weidman et al., 1987). Proteoliaisin-mediated insertion of ovoperoxidase into the nascent FE may be a pervasive feature of echinoid FE assembly, since antigenically related proteins have been found in four other species (Somers et al., 1989).

In this study, we examined the Ca²⁺-dependent interaction of proteoliaisin with ovoperoxidase in rotary-shadowed Pt replicas. Ovoperoxidase is a uniformly sized globular molecule approximately 14 nm in diameter, while proteoliaisin appears as a rod-shaped molecule ranging from 70 to 130 nm in length. Low concentrations of Ca²⁺ lead to binding of a single ovoper-
oxidase molecule to a distal region of proteoliaisin forming a structure resembling a lollipop. Using immunogold labeling, we also examined the assimilation of these two proteins into the FE in quick-frozen, deeply etched samples. Both proteins are abundant in the FE as early as one minute after fertilization, but by five minutes the labeling density is markedly reduced. We attribute this to masking of antigenic sites by the paracrystalline coat. Thus, the ovoperoxidase-proteoliaisin complex is not part of the paracrystalline coat, but instead resides within the central portion of the FE.

MATERIALS AND METHODS

Rotary shadowing
Purified proteoliaisin and ovoperoxidase were prepared as previously described (Deits et al., 1984; Weidman et al., 1985). These proteins were mixed with glycerol to give final concentrations of 50% glycerol at protein concentrations ranging from 30 to 200 µg/ml. Samples of the protein mixture were sprayed onto freshly cleaved mica squares using an artist’s air brush. Samples were then dried in vacuo at room temperature in a Balzers 400D freeze-etch unit and rotary-shadowed with Pt (8°) and carbon (75°). Replicas were cleaned with bleach, washed, floated onto 300-mesh copper grids and viewed in a Philips CM12 electron microscope.

Immunogold electron microscopy
Proteoliaisin and ovoperoxidase were immunolocalized using polyclonal rabbit antibodies (Somers et al., 1989). Eggs from Strongylocentrotus purpuratus were collected in Tropic Marin seawater, poured through 100 µm mesh nylon cloth several times to remove the jelly layer and stored at 16°C until ready for use. Sperm was collected ‘dry’ in plastic Petri dishes and kept on ice until immediately before fertil-
was obtained from replicas. The cast shape changes from rounded to the number of gold particles on casts and between casts, and the number \times 612,000 using a Micron microfiche reader. For each negative, the direct off negatives that were enlarged to a final magnification of taraldehyde. Eggs were quick-frozen, freeze-fractured, etched and obtain the average cylinder height (\( h \)). The area of the cast domain was defined as: 19.3 \( \mu m^2 \) × (number of casts \( n \)). The average three-dimensional cast area was estimated by assuming the average cylinder height \( h \) and the average diameter \( d \) of casts. The average three-dimensional cast area was estimated by the average cylinder height (\( h \)) and the average diameter (\( d \)) of casts, which are the area of the non-cast domain was defined as: 19.3 \( \mu m^2 \) – (number of casts \( n \) × \( r^2 \)), where \( r \) = average cast radius. To calculate the area of the cast domain, we took into account the three-dimensional nature of the casts. The distribution of proteoliaisin and ovoperoxidase label among the two assembly domains was quantitated by counting gold particles directly off negatives that were enlarged to a final magnification of \( \times 612,000 \) using a Micron microfiche reader. For each negative, the number of gold particles on casts and between casts, and the number of casts, were counted for each time point. We found that the presence of EGTA was essential for viewing proteoliaisin in a monomeric form. If this chelator was absent, the resulting proteoliaisin molecules had a tendency to self-aggregate, suggesting that trace amounts of Ca\(^{2+}\) may affect proteoliaisin structure. Since proteoliaisin is exposed to approximately 10 mM Ca\(^{2+}\) from seawater during FE assembly, we examined the effects of this cation on proteoliaisin structure. Proteoliaisin was mixed in distilled water containing either 7 mM EGTA, 50 \( \mu \)M Ca\(^{2+}\) or 10 mM Ca\(^{2+}\), and the lengths of the resulting molecules were measured.

These molecules appeared as uniformly sized globular particles approximately 14 nm in diameter (Fig. 1M).

In a few cases, we observed two ovoperoxidase molecules molecule bound to a distal region of proteoliaisin (Fig. 3A-K). We also observed larger, more complex aggregates along their lengths with ovoperoxidase molecules (Fig. 3M-N, arrows). Weidman et al. (1985) examined the interaction of ovoperoxidase with its intermediary protein, proteoliaisin (Weidman et al., 1985). To examine this interaction in rotary-shadowed replicas, ovoperoxidase and proteoliaisin were mixed in buffer containing 50 \( \mu \)M Ca\(^{2+}\). Under these conditions, we most frequently observed a single ovoperoxidase molecule bound to a distal region of proteoliaisin (Fig. 3A-K). In a few cases, we observed two ovoperoxidase molecules bound per proteoliaisin (Fig. 3L). When proteoliaisin and ovoperoxidase were mixed in buffer containing 500 \( \mu \)M Ca\(^{2+}\), proteoliaisin formed extended strands that were decorated along their lengths with ovoperoxidase molecules (Fig. 3M-N, arrows). We also observed larger, more complex aggregates under these conditions, but it was difficult to distinguish individual molecules within these aggregates.

### RESULTS

#### Ultrastructure of ovoperoxidase and proteoliaisin

The structure of proteoliaisin wasexamined in rotary-shadowed platinum replicas of samples sprayed onto a mica surface. To examine proteoliaisin structure in the absence of Ca\(^{2+}\), purified proteoliaisin was mixed in distilled water containing 7 mM EGTA prior to spraying onto mica. Under these conditions, proteoliaisin molecules appeared as discrete rod-like structures averaging 106 nm in length (Fig. 1A-J). A globular protrusion was observed at one end of many of the molecules (Fig. 1, arrows). Occasionally, non-standard lengths were observed, suggesting that proteoliaisin may form dimers (Fig. 1K) or even trimers (Fig. 1L). Rotary-shadowed views of purified ovoperoxidase molecules were distinctly different.
as a fibrous carpet punctuated with dome-shaped microvillar casts (Chandler and Heuser, 1980). Completed envelopes appear strikingly different, since the FE is entirely coated with highly organized rows of paracrystalline material both at the casts and in the regions in between (Chandler and Heuser, 1980). This process, which begins about 2 minutes after fertilization and is complete by 5 minutes, results in the transformation of the dome-shaped microvillar casts into tent-shaped structures (Chandler and Heuser, 1980). To examine the assimilation of ovoperoxidase and proteolaisin into the assembling FE and determine their relationship to the paracrystalline coat, eggs fixed at one, two and five minutes after fertilization were immunogold labeled and then examined with QF/DE/RS electron microscopy. By one minute after fertilization, we found that proteolaisin had been incorporated into the FE both at the casts and in the regions in between (Fig. 4A). However, the density of immunogold-labeled proteolaisin appeared to be much higher in the cast regions (Fig. 4A). Similarly, ovoperoxidase was detected in the cast and non-cast regions, with the highest densities observed in the cast regions (Fig. 4B). When

Fig. 3. Ca²⁺-dependent binding of ovoperoxidase to proteolaisin. (A-L) Gallery of ovoperoxidase molecules bound to proteolaisin monomers in the presence of 50 µM Ca²⁺. (M,N) Ovoperoxidase (arrowheads) bound to proteolaisin aggregates in the presence of 500 µM Ca²⁺. Bar, 0.1 µm.
Fig. 4. Immunolocalization of proteoliasin (A) and ovoperoxidase (B) in the fertilization envelope one minute post-insemination. The density of both of these antigens is higher in the cast domain than in the surrounding regions. (C) Immunocytochemical control. When rabbit IgG (1:40) was substituted for the primary antibody, virtually no non-specific labeling was detected. Bar, 0.1 μm.
rabbit IgGs were substituted for anti-ovoperoxidase or anti-
proteoliaisin, virtually no immunogold label was present either
on the casts or in the regions in between (Fig. 4C).

A nascent paracrystalline coat appeared on some envelopes
by one minute after fertilization (Fig. 5, below broken line). In
the regions where coating had begun, the quantity of proteoli-
aisin label was markedly reduced compared to surrounding
areas (Fig. 5). Similarly, in FEs fixed five minutes after fertil-
ization, which are entirely coated with paracrystalline material,
the density of proteoliaisin in the cast and non-cast regions was
greatly reduced from the quantity observed at one minute (Fig.
6A).

When ovoperoxidase activity is inhibited with ATA, the
casts become coated with paracrystalline material and undergo
a normal transition to tent-shaped; however, paracrystalline
coating is disrupted in the regions between casts (Mozingo and
Chandler, 1991). Since paracrystalline material is absent in the
non-cast regions, it might be expected that labeling density
would remain high, similar to one minute envelopes that are
also uncoated. However, we found that immunogold labeling
is reduced relative to one minute envelopes and similar to five
minute envelopes (Fig. 6B).

The distribution of proteoliaisin and ovoperoxidase label
among the cast and non-cast domains was quantitated by
counting gold particles directly off negatives (see Materials
and Methods for details). The density of immunogold labeled
proteoliaisin was greatest in both domains at one minute post-
inspemination and was reduced in both domains by five minutes
post-inspemination and when ovoperoxidase was inhibited (Fig.
7A). The immunogold distribution for ovoperoxidase was
similar to proteoliaisin except at lower density (Fig. 7B). This
may reflect a difference in sensitivity between the two anti-
bodies (Somers et al., 1989) rather than relative quantities of
the two antigens.

DISCUSSION

Ovoperoxidase is a 70 kDa heme glycoprotein that has been
localized to cortical granules prior to fertilization and to the FE
afterwards (Deits et al., 1984; Katsura and Tominaga, 1974;
Klebanoff et al., 1979; Somers et al., 1989). In this study, using
rotary shadowing, we have shown that ovoperoxidase is a
globular molecule approximately 14 nm in diameter. Proteoli-
aisin, the 230 kDa protein that mediates attachment of ovoper-
oxidase to the vitelline layer (Weidman et al., 1985), appears
as a rod-like structure approximately 106 nm in length, in the
absence of Ca\(^{2+}\). Similarly, Weidman et al. (1987) character-
ized proteoliaisin as a highly asymmetric protein using analyti-
cal ultracentrifugation. The molecular asymmetry of proteoli-
aisin is intermediate between those of the fibrous proteins
spectrin and fibronectin (Weidman et al., 1987). It has been
reported that proteoliaisin may self-associate and that the
presence of Ca\(^{2+}\) increases the degree of self-association
(Weidman et al., 1987). The present study indicates that pro-
telosialisin is capable of forming multimeric complexes, since
we observed long (>220 nm), complicated, interdigitating networks in the presence of 10 mM Ca\(^{2+}\).

Visualization of the ovoperoxidase-proteoliaisin interaction showed that a single ovoperoxidase molecule binds to a distal region of proteoliaisin when the [Ca\(^{2+}\)] = 50 \(\mu\)M. This is consistent with the observation that the binding domain for ovoperoxidase is near the amino-terminal end of proteoliaisin (Somers and Shapiro, 1991) and that the stoichiometry of the proteoliaisin-ovoperoxidase-binding interaction is 1:1 (Weidman et al., 1987). Increased Ca\(^{2+}\) concentrations (500 \(\mu\)M), led to the formation of extended proteoliaisin strands that were decorated along their lengths with ovoperoxidase molecules. The Ca\(^{2+}\)-dependent formation of proteoliaisin multimers, and the interaction of ovoperoxidase with these structures, may facilitate FE assembly by abating diffusion of these proteins away from the assembling structure. Indeed, structures resembling the ovoperoxidase-proteoliaisin complexes visualized here have previously been observed in early envelopes (Chandler and Kazilek, 1986).

The completed FE is a trilaminar structure consisting of a central layer of filaments sandwiched between thin layers of paracrystalline material (Inoue et al., 1967; Inoue and Hardy, 1971). We found that coincident with paracrystalline coating of the envelope, the quantity of ovoperoxidase and proteoliaisin immunogold label was markedly reduced (Figs 5 and 7) and attribute this to masking of antigenic sites by the paracrystalline protein coat. This suggests that the proteoliaisin-ovoperoxidase complex is assembled into the central core of the envelope, rather than the paracrystalline layer, which renders it inaccessible to the immunoreagents used in this study. Moreover, immunolocalization of proteoliaisin using thin-section electron microscopy, a technique in which immunoreagents have access to both FE layers, indicates that the central layer may be preferentially labeled (Somers et al., 1989).

FEs formed after 5 minutes in the presence of ATA showed a marked reduction in immunogold label compared to FEs at one minute post-insemination (Fig. 7). This result was unexpected; we predicted that the quantity of immunogold label would be similar, since both of these structures lack a paracrystalline protein coat. In addition, isolated FEs from eggs fertil-
ized in the presence of ATA (soft fertilization envelopes; SFE) are known to contain ovoperoxidase, since they can be hardened in vitro by removing the inhibitor and adding H₂O₂ (Kay et al., 1982). However, it has been pointed out that in vitro hardened SFEs are not identical to their in vivo counterparts (Kay and Shapiro, 1987). In vivo hardened FEs acquire approximately twice as much SDS-insoluble material during hardening compared to SFEs hardened in vitro (Kay and Shapiro, 1987). In addition, during ovoperoxidase-catalyzed iodination of proteins within the FE, in vivo hardened FEs incorporate more than twice the amount of ¹²⁵I into proteins compared to in vitro hardened SFEs (Kay and Shapiro, 1987). Thus SFEs do not incorporate as much ovoperoxidase as normal FEs, which may account for the reduction of immunogold label that we observed in this study. This suggests that ovoperoxidase activity may be necessary for the maintenance of a stable association between the nascent fertilization envelope and the proteoliaisin/ovoperoxidase complex or, alternatively, that ATA interferes with binding of the ovoperoxidase-proteoliaisin complex to the FE.

In a previous study, we found evidence for two assembly domains within the *S. purpuratus* FE, which could be distinguished by their enzymatic requirements for maturation (Mozingo and Chandler, 1991). Protease and transglutaminase activities were required for proper coating of the casts. Inhibition of these activities blocked coating and transformation of the casts into tent-shaped structures (also see Battaglia and Shapiro, 1987). In vivo hardened FEs acquire approximately twice as much SDS-insoluble material during hardening compared to SFEs hardened in vitro (Kay and Shapiro, 1987). In addition, during ovoperoxidase-catalyzed iodination of proteins within the FE, in vivo hardened FEs incorporate more than twice the amount of ¹²⁵I into proteins compared to in vitro hardened SFEs (Kay and Shapiro, 1987). Thus SFEs do not incorporate as much ovoperoxidase as normal FEs, which may account for the reduction of immunogold label that we observed in this study. This suggests that ovoperoxidase activity may be necessary for the maintenance of a stable association between the nascent fertilization envelope and the proteoliaisin/ovoperoxidase complex or, alternatively, that ATA interferes with binding of the ovoperoxidase-proteoliaisin complex to the FE.

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In this study, by quantifying the distribution of ovoperoxidase and proteoliaisin among the two domains, we found that ovoperoxidase is in fact abundant in the cast domain. This suggests that ovoperoxidase performs a function at the microvillar casts other than crosslinking paracrystalline material into the FE. Instead, at these sites, ovoperoxidase may crosslink substrates that reside within the central layer of the FE.

In *S. purpuratus*, secretory material isolated from cortical granules forms a crystalline precipitate in the presence of calcium (Bryan, 1970a,b). This material has an ultrastructural appearance identical to the paracrystalline coat present on completed envelopes and consists of at least five major proteins, of which only ovoperoxidase and proteoliaisin have been characterized (Bryan, 1970a; Kay et al., 1982; Weidman and Kay, 1986; Shapiro et al., 1989). This study clarifies the spatial organization of the ovoperoxidase-proteoliaisin complex within the FE and its relationship to the paracrystalline coat. Proteoliaisin and ovoperoxidase are assimilated into the central core of the envelope early in the assembly process, by one minute after fertilization (Fig. 8). Ca²⁺-dependent aggregation of proteoliaisin may contribute, in part, to the marked thickening of this layer that occurs within 3 minutes after fertilization (Carroll and Cohen, 1990; Chandler...
and Heuser, 1980). Next, paracrystalline protein is deposited on the surface of the FE and subsequently is crosslinked into the structure by ovoperoxidase (Fig. 8). The role of the remaining Ca2+-insoluble components in this scheme remains unresolved. Proteoliaisin has been shown to form Ca2+-stabilized complexes with two other components of uncross-linked FEs (Weidman et al., 1985), implicating proteoliaisin as a mediator of additional protein-protein interactions during FE assembly. Ultimately, it should be possible to define how all components of the cortical granule exudate are assembled and arranged in the completed fertilization envelope.

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REFERENCES


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