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Evidence that insect embryogenesis is regulated by ecdysteroids released from yolk proteins

(protein degradation/ecdysteroid conjugates/lipase/fatty acid binding)

MARY BOWNES*, ALAN SHIRRAS, MAIREAD BLAIR, JOHN COLLINS, AND ANDREW COULSON

Department of Molecular Biology, University of Edinburgh, King's Buildings, Mayfield Road, Edinburgh EH9 3JR, United Kingdom

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ABSTRACT **That the yolk proteins (YPs), or vitellins, stored in the oocytes of insects are a nutritional store for subsequent embryogenesis has long been assumed. Exhaustive data base searching programs revealed highly significant sequence similarity between the three YPs of *Drosophila melanogaster* and part of the triacylglycerol lipase of the domestic pig. Based upon time of degradation of YPs during embryogenesis, existence of maternally stored ecdysteroid conjugates in embryos, location of these conjugates in locust embryos, and the fact that free active ecdysteroid hormones are released at a specific time in embryogenesis to trigger cuticle deposition, we postulate that the similarity reflects a common property of *Drosophila* YPs—the ability to bind the fatty acid ecdysteroid conjugates. Our finding of conjugated ecdysteroids tightly bound to purified *Drosophila* YP supports this prediction.**

The three major yolk proteins (YPs) of *Drosophila* (1) are synthesized in the ovarian follicle cells and fat body (2–4) and selectively accumulated in developing oocytes. The YPs are assumed to play a nutritional role in embryogenesis; no studies have investigated other functions for the proteins.

The three polypeptides are each encoded by a single-copy gene located on the X chromosome (5, 6). We recently sequenced the gene coding for the smallest polypeptide, YP3 (7), which was then compared with the sequences of YP1 and YP2 (8, 9). They form a closely conserved family of genes with 56% similarity between YP1 and YP2, 56% similarity between YP1 and YP3, and 50% similarity between YP2 and YP3. The overall similarity between YP1, YP2, and YP3 is 43% and is particularly prominent in the C-terminal two-fifths of the polypeptides, where the similarity reaches 57%. Probably important conserved functional regions, such as sites recognized in receptor-mediated endocytosis, will be located in this region.

To search for potential functions for the YPs we compared their sequences with other sequenced proteins. Similarity with a lipase from the domestic pig was identified. From this potential homology we developed a model for a new function of YPs in embryogenesis. This model has been experimentally tested, and we now propose that degradation of the YPs leads to release of an ecdysteroid hormone that can trigger key events in embryogenesis, including secretion of the cuticle.

MATERIALS AND METHODS

Computation. The regions of sequence that showed the highest degree of local similarity when scored by the Dayhoff (10) similarity table for 100 accepted point mutations (PAMs) per 100 residues were found in an exhaustive search between each query sequence and the entire National Biomedical Research Foundation (NBRF) protein sequence data base, version 8 (809386-amino acid residues). The 4096 computer alignments yielding the highest degree of similarity, including gaps where necessary, are found by the program as implemented on an ICL computer (International Computers, Putney, London) 64 × 64 distributed array processor (11), and the significances of these results were analyzed by reference to the distribution of these results (12).

Purification of the YPs. Embryos were homogenized in 400 mM NaCl/20 mM $MgCl_2$/10 mM sodium orthophosphate, pH 7.0, and centrifuged. The supernatant was filtered, and phenylmethylsulfonyl fluoride was added to a final concentration of 100 μM. Ammonium sulphate was added slowly to saturation, and the extract was then cleared by centrifugation. The supernatant was used to prepare YPs by dialysis, after which the sample was freeze-dried.

Radioimmunoassay of Ecdysteroids. For each experiment a standard curve was prepared using [^{3}H]ecdysone and 20-hydroxyecdysone. RIA techniques are detailed in Smith and Bownes' work (13). The antiserum used was prepared by Briers and de Loof (14). Pig liver esterase (100 units; Sigma) was dissolved in 20 μl of 50 mM sodium acetate, pH 5.3. For each reaction we used a protease mixture containing type XXI protease from *Streptomyces griseus* (4 mg/ml), protease type IV from *Streptomyces caespitosus* (1 mg/ml), and trypsin type III from bovine pancreas (1 mg/ml) (all from Sigma). All samples were digested in 200 μl of sodium acetate, pH 5.3, 1 μl of protease mixture, and 20 μl of esterase suspension (except for one experiment where a higher concentration was used as described in *Results and Discussion*). Experimental samples and controls were mixed and incubated overnight at 37°C. Ecdysteroids were extracted with methanol, and RIAs were done as reported in ref. 13.

Antibody Precipitation of YPs. To remove traces of other proteins copurifying with the YPs in the above-described method, the YPs were precipitated in solution by an antibody that had been raised to the three yolk polypeptides cut from $NaDodSO_4$/polyacrylamide gel (15).

RESULTS AND DISCUSSION

To see whether any functions for the YPs might be detected by structural comparison with other known proteins, we used a distributed array processor to search for similarity to all protein sequences in release 8 of the NBRF protein sequence data base (10–12). Each YP sequence had the greatest similarity with the other YPs; YPs all shared highly significant sequence similarities with triacylglycerol lipase of the domestic pig, the only protein in the data base that showed significant similarity with all three YPs. The similarity covers long stretches of protein-sequence residues

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Abbreviation: YP, yolk protein.

*To whom reprint requests should be addressed.

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Lipase   95 KVESVNCICVDWKGGSR-TGYTQ-ASQNIRIVGAEVA-YFVEVLKSSLGYSPSNVHVIGHSLGSHAAGEAGR---R
YP 1    198 KTQSGDIIVIDL--GSKLNTYERYAMLDIEKTGAKIGKWIVQMVNE-LDMPFDTIHLIGQNVGAHVAGAAAQEFTR
YP 2    199 DTKTGDLIVIQL--GNAIEDFEQYATLNIERLGEIIGNRLVE-LTNTVNVPQEIIHLIGSGPAAHVAGVAGRQFTR
YP 3    207 KAASGDLIIIDL--GSTLTNFKRYAMLDVLNTGAMIGQTLID-LTNXKGVPQEIIHLIGQGISAHVAGAAGNKYTA
              k--s-*-I-*d---Gs----*---A--*i---Ga-**--*v*-l----------*H*IG----*H*AG-Ag----r

          -TNG-TIERITGLDPAEPCFQGTPE--LVRLDPSDAKFVDVIHTDAAPIIPNLGFGMSQTVGHLDFFPNG-GKQMPG 236  Lipase
          LT-GHKLRRVTGLDPSKIVAKSKNT--LTGLARGDAEFVDAIHT-S--VY-GMGTPI-RS-GDVDFYPNGPAAGVPG 338  YP 1
          QT-GHKLRRITALDPTKI-Y-GKPEERLTGLARGDADFVDAIHT-SA--Y---GMGTSQRLANVDFFPNGPSTGVPG 339  YP 2
          QT-GHKLRRITGLDPAKV-LSKRPQ-ILGGLSRGDADFVDAIHT-ST--F-AMGTPI-R-CGDVDLYPNGPSTGVPG 347  YP 3
          -T-G--*-RiTgLDPa-------p---L--L---DA-FVD*IHT-*-------G-------g-*Df*PNG----*PG
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FIG. 1. Similarity between triacylglycerol lipase and YP. Capital letters refer to amino acids that are identical in all four proteins. Lower-case letters are shown when two of the three YPs have an identical amino acid to that of the lipase. ★, Conservative substitutions with reference to the 100 accepted point mutations per 100 residues table of Dayhoff (10). Numbers indicate amino acid positions in proteins.

81–230 of the lipase aligning with residues 186–331 of YP1, residues 108–241 of lipase aligning with residues 211–344 of YP2, and residues 95–268 of lipase aligning with residues 207–386 of YP3. The local alignment leading to the highest degree of similarity between YP1 and triacylglycerol lipase received a score of 170, a figure expected by chance only once in a search of 100,000 data bases of this size containing unrelated protein sequences. The score between YP2 and YP3, respectively, and the lipase was 213 and 186, scores expected once in 80,000,000 and 5,000,000 searches of data bases of this size, respectively. Similar regions of the YPs are thus related to a single region of the lipase (Fig. 1).

The 449 residues of the triacylglycerol lipase secreted by the pig pancreas have been sequenced (16–19), and some studies have been made of the substrate binding site (19). The active site is thought to include residues 147–156, which include the sequence Ile-Gly-His-Ser-Leu-Gly-Ser-His. Ser-152 appears to be important in lipid–water interface recognition. Although the similar region of the YPs includes the active site, the essential serine residue is not conserved.

Purified YPs failed to act as a lipase under conditions where the triacylglycerol lipase was active. Lipase activity was determined by measuring the release of [1-^{14}C]-palmitate from glycerol tri-[1-^{14}C]palmitate (Amersham). Under the chosen assay conditions, activity was detectable in purified pig pancreatic lipase and whole adult fly extracts but was not detectable in the purified YP preparation; this suggests that the YPs do not function as lipases.

Why then would they be structurally similar to a lipase? Possibly they bind a molecule similar to triacylglycerol.

Ecdysteroids are the molting hormones of insects, being released before synthesis of the cuticle (20, 21). In many insects including *Drosophila*, ecdysteroids are stored in the embryos as conjugates (22–24) of both low and high polarity (23–25), and some apolar molecules have been identified as long-chain fatty acid esters of the ecdysteroids. Such storage forms exist in other arthropods (25) and appear to have roles in regulating release of free ecdysteroids to control reproduction and development. The high-polarity conjugates are digestible with *Helix pomatia* hydrolases—e.g., β-glucuronidase—and the low-polarity conjugates are digestible with pig liver esterase (26).

In several insect embryos ecdysteroids are released before cuticle secretion (26, 27), and *Drosophila* embryos are no exception. Kraminsky *et al.* (28) showed that free ecdysteroid was released before cuticle secretion in the embryo. In locusts polar conjugates are bound to vitellin (29), and as the vitellin is degraded, peaks of free ecdysteroid hormone appear that coincide with secretion of cuticles (30).

We propose, therefore, that the similarity with the triacylglycerol lipase reflects binding-site similarities and that the function is to bind apolar conjugates of ecdysteroids. Because YPs are proteolytically cleaved during embryogenesis, the conjugates would become available for metabolism and subsequent release of hormone. Thus, the YPs

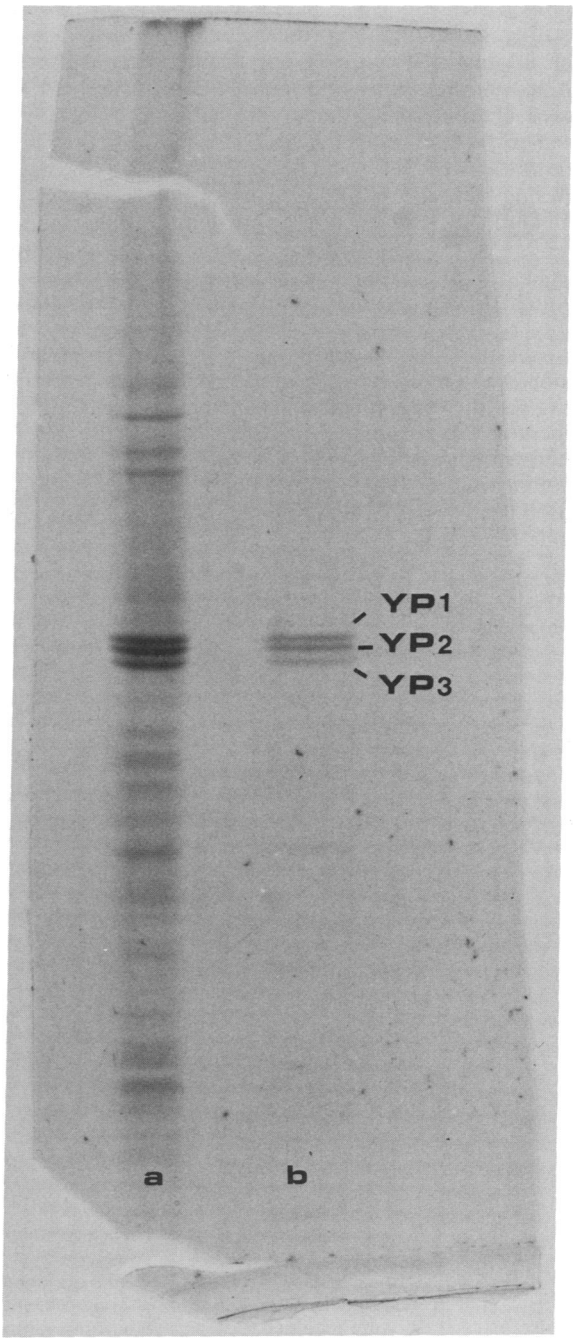

FIG. 2. Purification of the YPs. Polypeptides were separated by $NaDodSO_4$/gel electrophoresis and stained with Coomassie blue. Lanes: a, starting material, *Drosophila* embryos 0–12 hr after deposition; b, after purification of YPs.

would be important molecules in embryogenesis, their timed breakdown leading to a timed release of hormone.

If this hypothesis is correct, apolar conjugates of ecdysteroids should be tightly bound to the YPs. Using the procedure shown in the legend for Fig. 2 we purified the YPs (Fig. 2). We then treated these purified proteins with protease, pig liver esterase, and a combination of both enzymes. The resulting samples were extracted with methanol and subjected to an RIA to detect free ecdysteroids.

Ecdysteroids were released, confirming that they had been bound to the YPs. They were released most effectively by the protease and esterase acting in concert, suggesting that degradation of the YP is important in presenting the conjugates to the esterase. We obtained more ecdysteroids by adding the esterase concomitantly with the protease, rather than by adding the protease before the esterase.

One interesting feature of our results was the fact that when more YPs were present, fewer ecdysteroids were released. Perhaps insufficient protease and esterase are present to release all of the ecdysteroids, and thus higher concentrations of YPs could be inactivating the enzymes, or interfering with access to their correct functional locations. Results in Fig. 3, as well as showing that this phenomenon is repeatable, show that higher concentrations of enzymes do release more free hormone, suggesting that, indeed, too little enzyme had been available to release all YP-bound ecdysteroids.

To ascertain that this hormone was released from the YPs and not from a molecule that had copurified, we precipitated the YPs with an antibody made against the three yolk polypeptides cut from a $NaDodSO_4$/acrylamide gel. This method would remove any copurifying proteins of dissimilar molecular weights. Fig. 3 shows that free ecdysteroids were released from these antibody-precipitated YPs by the mixed-enzyme treatment.

How many molecules of YPs are necessary to bind one conjugated ecdysone molecule is unclear. If 1 mol of YP binds 1 mol of ecdysone, then 1 mg of YP should bind 10 μg of ecdysone. We detect much less than this amount, but incomplete degradation by the protease and esterase, loss of bound molecules during YP purification, and contaminating salts and proteins could all lower the amount found experimentally. It is also unlikely that every molecule of YP binds a molecule of ecdysone—this would release far too much ecdysone into the developing embryo. Several YP molecules may cooperate to bind one conjugated ecdysteroid molecule, or a fixed quantity of conjugated ecdysteroids may be produced (probably in the follicle cells) and associate with the YPs in that egg.

Vitellogenins are probably important in regulating embryogenesis by binding molecules for subsequent timed release. This fundamental process may have been carefully conserved during evolution because it is very similar in *Drosophila* and *Locusta*, and storage forms of ecdysteroids exist in embryos of many insects and arthropods. Whether there are binding sites for polar ecdysteroid conjugates on *Drosophila* YPs, as well as apolar ones, is unclear. The β-glucuronidase samples purified from snails (which carry ecdysteroids), which we obtained, gave positive values in the RIA and, therefore, could not be used to test ecdysteroid release from the YPs. However, note that this reinforces our finding that ecdysteroids are genuinely bound to the YPs. Snail β-glucuronidase has ecdysone as a contaminant. Methanol extraction of contaminated β-glucuronidase removes ecdysone, which is therefore free ecdysone. Bound ecdysone associated with YPs can only be released, as we have demonstrated, by protease and esterase treatment.

Recently apolar conjugates have been found to be much more abundant in *Drosophila* females than males (G. Kaufmann, U. Schweizer, and A. Dübendorfer, personal communication). Because the YPs are found only in females and the storage of hormones in eggs is limited to females, further independent evidence favors our model. The nature of the binding and the identification of the conjugates remain to be studied.

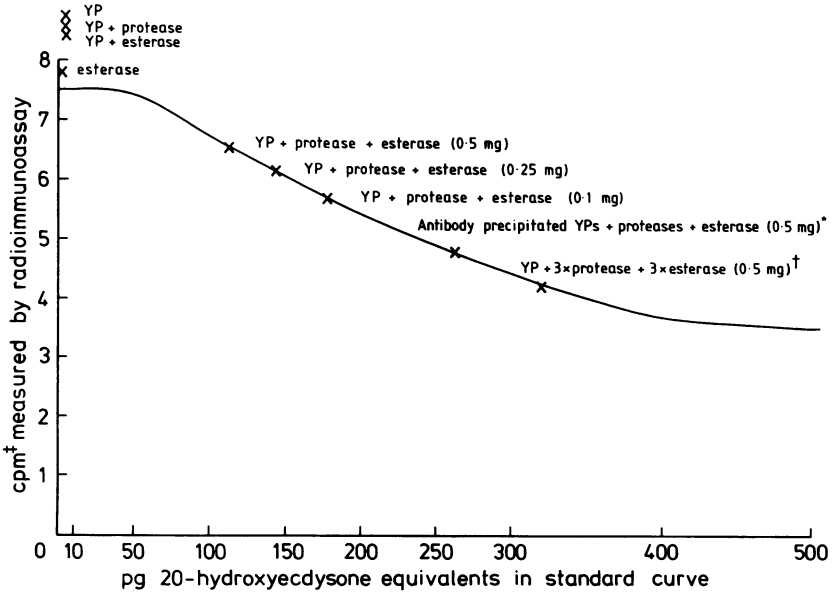

FIG. 3. Ecdysteroids released from purified YPs are detectable by RIA. All experimental samples were digested with 200 μl of sodium acetate, 1 μl of protease cocktail, and 20 μl of esterase (except for those marked by *). Controls of esterase alone and YPs treated with either protease or esterase were included. After overnight incubation ecdysteroids were extracted and subjected to RIA. (For each experiment a separate standard curve was prepared.) The results of one experiment are marked on the appropriate standard curve. Similar results have been obtained in three separate experiments as described in *Results and Discussion*.

*The YPs were solution-antibody precipitated before digestion as in ref. 15.

†Three times concentration of protease and esterase were added.

‡cpm $\times 10^{-3}$.

In conclusion, an unexpected sequence similarity between the YPs and a lipase led us to devise and successfully test a model in which YPs play an important role in regulating embryogenesis; this mechanism seems to have been conserved in many insects and arthropods.

Note Added in Proof. A cDNA encoding a lipase from the rat liver has been cloned and sequenced (31). This also has sequence similarity to the three YPs extending from amino acid 90 in the rat hepatic lipase to amino acid 231 using the alignments of the pig lipase and the three YPs shown in Fig. 1. Particularly well conserved are the blocks RiTgLDPa, DA-FVD★IHT-★, and ★Df★PNG (see Fig. 1). This adds weight to the idea that the YPs can bind lipid molecules either as triacylglycerol itself, or as lipids conjugated to ecdysteroids, as suggested in this paper, or both.

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