Control of Juvenile Hormone Biosynthesis

EVIDENCE FOR PHOSPHORYLATION OF THE 3-HYDROXY-3-METHYLGLUTARYL COENZYME A REDUCTASE OF INSECT CORPUS ALLATUM*

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The activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase, a key enzyme of juvenile hormone biosynthesis, has been measured in the supernatants of homogenates (10,000 × g) prepared from the corpora allata of the adult tobacco hornworm moth, Manduca sexta. Enzyme activity was inhibited 80% by 50 mM NaF, a known phosphoprotein phosphatase inhibitor, if present during extirpation of the glands and all subsequent workup of the tissue. Reductase activity was also significantly decreased (20–30%) in homogenates preincubated with 4 mM MgCl2 and 2 mM ATP.

These results provide evidence that reductase in the insect undergoes phosphorylation and dephosphorylation similar to that occurring with reductase of mammalian liver. If so, this would provide a rapid method for modulating juvenile hormone synthesis.

Insect juvenile hormones are sesquiterpenoids produced in the corpora allata, a small pair of glands posterior to the brain. These compounds play important roles in the maintenance of the larval form and in maturation of the reproductive system often reach immeasurably low levels prior to metamorphosis (1). Production of juvenile hormone is believed to be controlled by feedback pathways (2) and by neural and humoral signals (3), and under certain circumstances, to be affected by the steroid molting hormone, 20-hydroxyecdysone (4).

The pathway for JH biosynthesis shares common intermediates with sterol biosynthesis, and may share some aspects of the control mechanisms which operate on the sterol pathway. However, insects do not synthesize sterols (5) and juvenile hormone synthesis is, in fact, altered by both Mg-ATP and F- in much the same way as the liver enzyme. We report here the results of these investigations.

MATERIALS AND METHODS

Animals—Colonies of M. sexta were raised as previously described (13). Eggs were generously provided by Dr. J. P. Reinecke, U. S. Department of Agriculture, Fargo, ND. Unfed adult animals were used 0–4 days after time of eclosion.

HMG-CoA Reductase—CA homogenates were assayed for HMG-CoA reductase activity as described by Kramer and Law (14) with the following modifications. CC-CA complexes were rinsed free of dissecting saline (16) by three 100-pl-washes with a buffer containing 50 mM KH2PO4, 200 mM KCl, 5 mM dithiothreitol, 5 mM EDTA, and 0.25% Kryo ROB, pH 7.4 (17). MgCl2 and CaCl2 were omitted from this dissecting saline if 50 mM NaF was present. In experiments involving the addition of 2 mM ATP and 6 mM MgCl2, the phosphate buffer contained 1 mM EDTA, rather than 5 mM EDTA. The washed CC-CA glands (20–30) were homogenized in 70 µl or 100 µl of the above buffer, blended with a Vortex mixer, and warmed at 37 °C for 10 min. Tissue homogenates were centrifuged at 10,000 × g and 23 °C for 10 min in a water-cooled microcentrifuge (Misco, Berkeley, CA).

HMG-CoA reductase activity was assayed by a modification of the method of Shapiro et al. (18). For each incubation, all stock solutions were adjusted to pH 7.4 with KOH before addition to the homogenate. Assays were initiated by the addition of a cofactor-substrate solution containing 450 nmol of NADP, 4.5 µmol of glucose-6-phosphate, 0.67 IU of glucose-6-phosphate dehydrogenase, and 20 µmol of DL-[methyl-
Juvenile Hormone Biosynthesis

HMG-CoA (specific activity, 11–12 dpm/pmol). Incubations were carried out at 37 °C for 90 min in sealed Reacti-Vials housed within a Temp-Blok module heater shaking at 170 rpm. Reactions were terminated by the addition of 25 μl (5 μl for incubating volumes greater than 250 μl) of 6 n HCl, followed by 1020 or 1160 dpm of [2-14C]mevalonolactone (specific activity, 18 Ci/mol) as an internal standard and warmed at 40 °C for 30 min with shaking. Corrected recovery of the lactone typically ranged from 85–95%. Each sample was applied to two Silica Gel G thin layer chromatography plates and developed in benzene/acetone (1:1, v/v). Mevalonolactone was recovered in the region Rf 0.36–0.69 by scrapping the silica gel into 10 ml of toluene/ethanol/Omnifluor (21:12, v/v/w). In all cases, a sample of each CC-CA homogenate was treated with f + HCl before addition of the cofactor-substrate solution to serve as a denatured control. Enzyme activities are expressed as picomoles of mevalonate synthesized/h/CA gland pair equivalent.

Materials—[Methyl-3H]HMG-CoA (11.7 Ci/mmol) was obtained from New England Nuclear and [2-14C]mevalonolactone was purchased from Amersham Corp. Glucose-6-phosphate dehydrogenase (from bakers’ yeast) and glucose-6-phosphate, NADP, ATP, and HMG-CoA were all purchased from Sigma. Kyro EOB was supplied by Dr. R. D. Drotman, courtesy of Dr. R. B. Proctor and the Proctor Co. Kyro EOB is a synthetic nonionic detergent which has been used to solubilize plasma membranes, but not endoplasmic reticulum (17).

RESULTS

The CA is a minute organ (one CC-CA pair weighs ~20 μg, wet weight) and the amount of HMG-CoA reductase present is correspondingly small. Hence, activity measurements must be conducted over 1.5 h in order to obtain meaningful values for a small number of glands (3–6 CA pair equivalents/incubation). Mevalonolactone production has been shown to be linear for at least two h under the assay conditions. Extirpation of CA is practical only when the closely adhering CC and a portion of the aorta are also included. Measurement of reductase activity in CC and in aorta showed values representing only 6 and 2% of that measured in the CA, respectively. Therefore, the three tissues were homogenized together.

Nearly a 2-fold (70–95%) enhancement in reductase activity was observed when 0.25% (v/v) of the detergent Kyro EOB, was added to the homogenates. This was determined by homogenizing only the right CA of each gland pair in 0.25% Kyro EOB, and using the left CA to serve as a nondetergent control, thereby avoiding animal-animal variation. This detergent has been used by other investigators to enhance reductase activity in preparations of mammalian tissue.

TABLE I

| Incubation number | Nucleotide addition | Final EDTA concentration in reductase assay | % of Mg-CTP control ± S.D. |
|-------------------|---------------------|------------------------------------------|---------------------------|
| 1                 | Buffer control      | Male 99.9 ± 1.3 105 ± 7.2 100 ± 7.1     | 1 vs 2; N.S.,* p > 0.2    |
| 2                 | Mg-CTP control      | Male 100 ± 1.7 100 ± 7.1                | 3 vs 1; 3 vs 2; p < 0.001 |
| 3                 | Mg-ATP              | Male 75 ± 2.7 80 ± 5.8                   | 4 vs 1; 4 vs 2; 4 vs 3; p < 0.001 |
| 4                 | Mg-ATP              | Male 61.3 ± 1.7 64 ± 2.1                 | 5 vs 6; N.S., p > 0.2     |
| 5                 | Buffer control      | Female 101.6 ± 2.0 98 ± 8.6             | 7 vs 5; 7 vs 6; p < 0.001 |
| 6                 | Mg-CTP control      | Female 100 ± 2.8 100 ± 6.7              | 8 vs 5; 8 vs 6; p < 0.001 |
| 7                 | Mg-ATP              | Female 71.8 ± 3.0 75 ± 4.6               | 8 vs 7; N.S., p > 0.2     |
| 8                 | Mg-ATP              | Female 73.0 ± 1.6 74 ± 5.9               |                           |

* N.S., not significant.
Control of Juvenile Hormone Biosynthesis

Effects of NaF on HMG-CoA reductase activity

In A, male or female CA were homogenized in buffer (125 µl) and aliquots (25 µl) were taken in duplicate. These were diluted with either 25 µl of 100 mM NaF or additional buffer to serve as controls. In B, male or female adults were decapitated, a section of the exoskeleton was removed from the heads to expose the CA, and the heads were placed in either control dissection saline or in saline supplemented with 50 mM NaF. NaF was also present in the homogenization buffer and in the cofactor-substrate solution to maintain test tissues at 50 mM NaF throughout the experiment.

| Incubation | Number of assays (n) | Sex | Number of CA grains/µg | HMG-CoA reductase activity | Change in activity |
|------------|----------------------|-----|------------------------|-----------------------------|-------------------|
| A. NaF (50 mM) added to CA immediately after homogenization | 4 | Male | 4.40 | 125.2 ± 0.5 | 9.9 |
| With NaF | 4 | Male | 4.40 | 112.7 ± 1.3 | 7.1 |
| Control | 4 | Female | 3.40 | 115.9 ± 4.6 | 7.1 |
| With NaF | 4 | Female | 3.40 | 107.5 ± 2.2 | 7.1 |
| B. NaF (50 mM) added to CA before extirpation of glands | 6 | Male | 3.00 | 129.0 ± 6.7 | 80.1 |
| With NaF | 6 | Male | 2.75 | 27.6 ± 1.3 | 78.6 |
| Control | 6 | Female | 3.50 | 116.8 ± 2.6 | 78.6 |
| With NaF | 6 | Female | 3.25 | 23.2 ± 0.8 | 80.1 |

prepared dissection saline buffer containing 50 mM NaF and repeated the experiments with fluoride present at all times up to and during measurement of activity. The results shown in Table II (B) demonstrate a dramatic reduction in reductase activity in both sexes when F⁻ is continuously present. This can be explained by the continuous inhibition of phosphatase with no inhibition of kinase activity until after extirpation of the CA and subsequent homogenization in the presence of EDTA has occurred.

DISCUSSION

The results reported here show that HMG-CoA reductase of M. sexta CA responds to Mg-ATP and F⁻ in a manner quite analogous to the reductase of mammalian liver. This implies that the enzyme of CA exists in two forms, an active, nonphosphorylated form, and an inactive, phosphorylated form which are interchangeable by a protein kinase and a phosphoprotein phosphatase. Of course, confirmation of this hypothesis must await more definitive experiments, e.g. demonstration of the transfer of labeled phosphate from ATP to the enzyme as has been done with liver reductase. The difficulty in obtaining the required amount of purified enzyme from the minute CA will delay such work, however.

If this scheme is correct for the insect CA, then it would seem that both reductase kinase and phosphoprotein phosphatase levels are about equal in CA homogenates prepared from adults of either sex. It is impossible at this stage to decide what the actual ratios of active and inactive forms of reductase are in the intact CA of the animals. It is of interest that we were able to show earlier (14) that CA of adult males, unlike those from adult females, produced no JH in maintenance culture, while CA homogenates had reasonable HMG-CoA reductase levels. It may be that extirpation and subsequent maintenance of intact male CA resulted in rapid inactivation of the reductase through activation of the kinase, while in homogenates that contained EDTA the phosphatase reactivated the phosphorylated reductase. Another possible explanation is that derenervation of the CA in males stimulates some alternate isoprenoid synthetic pathway beyond phophoarcarvionate and this shunts intermediates away from juvenile hormone biosynthesis. Further experimentation is necessary to determine the nature of these sex differences. Whatever the reason, it appears that phosphorylation-dephosphorylation may provide rapid modulation of JH biosynthetic capacity in insects of both sexes.

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D J Monger and J H Law

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