Regulation of Ecdysteroid Signaling: Cloning and Characterization of Ecdysone Oxidase, a Novel Steroid Oxidase from the Cotton Leafworm, *Spodoptera littoralis*

Hajime Takeuchi, Jian-Hua Chen, David R. O’Reilly†, Philip C. Turner, and Huw H. Rees†

Cellular Regulation and Signaling Division, School of Biological Sciences, University of Liverpool, Life Sciences Building, Crown Street, Liverpool, L69 7ZB, U.K.

†Department of Biology, Sir Alexander Fleming Building, Imperial College of Science, Technology and Medicine, Imperial College Road, South Kensington, London, SW7 2AZ, U.K.

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†To whom correspondence should be addressed:
Tel: +44 151 794 4352; Fax: +44 151 794 4349; email: reeshh@liv.ac.uk
SUMMARY

One route of inactivation of ecdysteroids in insects involves ecdysone oxidase-catalyzed conversion into 3-dehydroecdysteroid, followed by irreversible reduction by 3DE 3α-reductase to 3-epiecdysone. We have purified from Spodoptera littoralis the first ecdysone oxidase and subjected it to limited amino acid sequencing. A reverse-transcriptase PCR-based approach has been used to clone the cDNA (2.8 kb) encoding this 65 kDa protein. Northern blotting showed that the mRNA transcript was expressed in midgut during the prepupal stage of the last larval instar at a time corresponding to an ecdysteroid titer peak. Conceptual translation of the ecdysone oxidase cDNA and database searching revealed that the enzyme is an FAD flavoprotein which belongs to the Glucose-Methanol-Choline (GMC) oxidoreductase superfamily. Ecdysone oxidase represents the only oxidase in eukaryotic animals known to catalyze oxygen-dependent oxidation of steroids; by contrast, oxidation of steroids in vertebrates occurs via NAD(P)⁺-linked dehydrogenases. The injection of RH-5992, an ecdysteroid agonist, induced the transcription of ecdysone oxidase, suggesting that ecdysone oxidase is an ecdysteroid responsive gene. The gene encoding this enzyme, consisting of five exons, has also been isolated. Sequences similar to the binding motifs for Broad-Complex and FTZ-F1 have been found in the 5′-flanking region. Southern blotting indicated that ecdysone oxidase is encoded by a single copy gene. We have determined the kinetic characteristics of this novel recombinant ecdysone oxidase produced using a baculovirus expression system.
INTRODUCTION

Molting and aspects of reproduction in insects are regulated by the steroidal molting hormones (ecdysteroids) (1). In immature stages of insects, the prothoracic glands are the major source of ecdysteroids, generally ecdysone (E\(^1\)), in most species. However, in most Lepidoptera examined, the major products of the glands are 3-dehydroecdysone (3DE), accompanied by varying proportions of ecdysone (2-6). The observation that interconversion of ecdysone and 3DE by prothoracic glands is not detectable in the cotton leafworm, *Spodoptera littoralis*, suggests that 3DE is likely to be an independent product of pathways of ecdysteroid biosynthesis in the glands (6). Following secretion, 3DE undergoes reduction to ecdysone by NAD(P)H-linked 3DE 3β-reductase in the hemolymph (3, 4, 6, 7). Ecdysone then undergoes 20-hydroxylation in certain peripheral tissues, yielding 20-hydroxyecdysone, which is considered to be the major active molting hormone in most insect species (8).

At specific stages in development, the ecdysteroid titer exhibits obligatory, distinct peaks (9). In immature stages, these result from increased ecdysteroid synthesis in the prothoracic glands, whereas decreases in titer arise from elevated ecdysteroid inactivation reactions in conjunction with enhanced excretion. Several transformations contribute to ecdysteroid inactivation (8), including the formation of 3-epi (3α-hydroxy) ecdysteroids, which are regarded as hormonally inactive (11-13). Production of 3-epiecdysteroids occurs in many insect orders, but appears to be prominent in lepidopteran midgut cytosol and entails conversion of ecdysteroid into 3-dehydroecdysteroid, followed by NAD(P)H-dependent irreversible reduction to 3-epiecdysteroid (Fig. 1, 8, 12-16). These reactions occur with both ecdysone and 20-hydroxyecdysone. Ecdysone oxidase (EC 1.1.3.16) is the enzyme which catalyzes the oxidation of ecdysteroid, and was first demonstrated, characterized and extensively purified from the blowfly, *Calliphora vicina* (17, 18).

However, the 3-dehydroecdysteroid can also undergo NAD(P)H-dependent 3DE 3β-reductase-catalysed reduction back to ecdysteroid (for reviews see 8, 19). The
significance of such competitive reaction between ecdysone oxidase and 3DE 3β-reductase is uncertain. Furthermore, particularly puzzling is the occurrence of enzymes for reversible interconversion of ecdysteroid and their 3-dehydro-derivatives in tissues of several species that are incapable of producing 3-epiecdysteroids (12, 19).

As part of our studies aimed at elucidating the regulation of ecdysteroid titer, including the reactions involved in ecdysteroid inactivation in *S. littoralis*, we have cloned and characterized the cDNA encoding the 3DE 3β-reductase (hemolymph) (20) and 3DE 3α-reductase (Malpighian tubules) (16). Furthermore, we have purified ecdysone oxidase from *S. littoralis* midgut plus attached Malpighian tubules and evidence suggests that the native enzyme consists of a trimer with apparent molecular mass of approximately 190 kDa and subunit molecular mass of approximately 64 kDa (21). Amino acid sequences of the NH₂-terminus as well as of interior tryptic peptides of the purified enzyme have been determined.

We now report the molecular cloning, characterization and heterologous expression of the cDNA encoding ecdysone oxidase of *S. littoralis*, together with analysis of the organization of the corresponding gene and its promoter. Conceptual translation and amino acid sequence analysis indicates that ecdysone oxidase belongs to the Glucose-Methanol-Choline (GMC) oxidoreductase superfamily. In fact, the ecdysone oxidase is novel in being, hitherto, the only eukaryotic animal steroid-metabolizing oxidase enzyme to be reported and characterized at the molecular level. By contrast, oxidation of steroids in vertebrates occurs via NAD(P)⁺-linked dehydrogenases.
EXPERIMENTAL PROCEDURES

Protein Sequence — Ecdysone oxidase from *S. littoralis* was purified as described previously (21), subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% gel, electrotransferred to ProBlott™ membrane, and visualized by Coomassie staining. A single band was observed, which was excised and sequenced by an automated pulsed liquid-phase sequencer (Applied Biosystems 471A) giving the NH₂-terminal amino acid sequence as XYAVGGXAGAGPAATYVA (where X represents an unidentified amino acid). To obtain sequences of the interior region of the enzyme, the purified protein was excised from an SDS-PAGE gel and cleaved with trypsin. The resulting tryptic peptides were purified by high-performance liquid chromatography and sequenced. The best internal sequence was: ETPYWXFTTIXXGVT.

cDNA Cloning and Sequencing — A PCR-based cloning strategy was used to isolate a cDNA fragment encoding the region between the two peptide sequences described above. Four degenerate primers were synthesized. Primer EO3-3 and EO-5 were designed on the basis of a part of the NH₂-terminal amino acid sequence (EO3-3, 5'-GTN GGN GCI GGI CCI GC, where I represents inosine, N is A/T/C/G; EO-5, 5'-GCN GGI CCN GCI GCN ACN TAY GT, where Y represents T/C); primers EO-AS3-2 and EO-AS3-3 were designed based on the internal proteolytic peptide fragment (EO-AS3-2, 5'- ATI GTI GTR AAI NIC CAI NIR TAI GG, where R represents A/G; EO-AS3-3, 5'- GTI GTR AAI NIC CAI NIR TAI GGI GT).

Total RNA was extracted using TRIzol (Life Technologies, Ltd.) from midgut dissected from larvae at 18 h into the last larval instar. First strand cDNA was reverse transcribed from the total RNA using a 1st Strand cDNA Synthesis Kit (Roche Molecular Biochemicals) with random primer p(dN)₆ supplied with the kit. cDNA synthesized with random primer served as template for PCR in which the above degenerate primers were used. PCR was carried out as follows: 1 cycle of 94°C for 3 min and 35 cycles of 94°C for 1 min, 47°C for 1 min, 72°C for 3 min and 1 cycle of
72°C for 7 min using EO3-3 and EO-AS3-2 primers. This PCR product was used as template for the nested PCR, which was carried out as follows: 1 cycle of 94°C for 3 min and 30 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 3 min and 1 cycle of 72°C for 7 min using EO-5 and EO-AS3-3 primers. The nested PCR yielded a product of approximately 300 bp.

The purified PCR product was cloned into pGEM®-T Easy Vector (Promega, Ltd.). Transformants were screened by colony PCR using M13 forward and reverse primers (5'- GTA AAA CGA CGG CCA G and 5'- CAG GAA ACA GCT ATG AC, respectively), and those showing the correct size of inserts were propagated in LB broth containing 100 µg/ml of ampicillin and plasmid DNA was purified after 16 h incubation at 37°C. Double-stranded DNA sequencing was performed by the dideoxy termination method using Sequenase Version 2.0 (USB™, Amersham Pharmacia Biotech, Ltd.). The sequences of three independent clones were compared to detect errors that could have occurred during the reverse-transcription and PCR amplification.

5'-Rapid Amplification of cDNA Ends (5'-RACE) — 5'-RACE was carried out to obtain the 5'-end of the cDNA. For this, mRNA from total RNA was isolated using a Dynabeads mRNA Purification Kit (Dynal Ltd., UK). A 5'-RACE System, Version 2.0 (Life Technologies, Ltd.) was used to amplify the 5'-terminus of the message for sequencing. Briefly, a gene-specific primer 1 (5'- CTT TCA AGG TTT GTC TTA A), designed on the basis of the sequence of the PCR product was hybridized to the mRNA and cDNA was synthesized using SUPERSCRIPT™ II reverse transcriptase. The RNA was then degraded with RNase mix (RNase H and RNase T1), and the cDNA was purified using a GlassMax spin cartridge supplied with the kit. A poly(dC) tail was added to the 3' terminus of the purified cDNA using dCTP and terminal deoxynucleotidyl transferase, and the cDNA region corresponding to the 5'-end of the mRNA was amplified by two successive rounds of PCR using additional gene-specific primers 2 and 3 (5'- CCG GTA CAA TGC TCT CCT and 5'- GGT TCG GTC CTG CTT CTA, respectively), together with the anchor primers supplied by the
manufacturer. The second round PCR yielded a product of approximately 400 bp, which was cloned into pGEM®-T Easy Vector, and the nucleotide sequences of several clones determined.

Construction and Screening of the Genomic Library — The genomic library was constructed using a Lambda FIX II/XhoI Partial Fill-In Vector Kit (Stratagene). The genomic DNA was prepared from the fat body and Malpighian tubules of S. littoralis in the last larval instar using a modified phenol extraction method as described in (22). It was partially digested with Sau3AI and the first two nucleotides of the 3' termini filled in. The genomic DNA fragments were ligated into the Lambda FIX II arms and packaged with Gigapack III Gold Packaging Extract (Stratagene).

Hybridization to the genomic library was performed for 18 h at 40°C in 6 x SSC (1 x SSC is 0.15 M NaCl, 0.015 M sodium citrate), 5 x Denhardt's reagent, 0.5% SDS, 100 µg/ml denatured, fragmented salmon sperm DNA, and a 32P-labeled DNA fragment produced using EO-5 and EO-AS3-3 primers as a probe. Membranes were washed at low stringency (2 x SSC, 0.1% SDS, at 40°C). The positive clones were analyzed by restriction digestion. The restriction fragments were subcloned into pBluescript II KS+ (Stratagene) and sequenced.

Determination of Transcription Initiation Site — The transcription initiation site of ecdysone oxidase mRNA has been determined using the solid phase CapFinder approach (23). mRNA from total RNA was isolated using a Dynabeads mRNA Purification Kit, and used to synthesize a solid-phase cDNA library with CapFinder Primer (5'- AAG CAG TGG TAT CAA CGC AGA GTG GCC ATT ATG GCC GGG). The RNA was then degraded with RNase H, and the immobilized cDNA was purified by using a magnet. The cDNA region corresponding to the 5'-end of the mRNA was amplified by two successive rounds of PCR using gene-specific primers 4 and 5 (5'- GTT CGG TCC TGC TTC TAG CAG CA and 5'- CAC GCT GAA GCG GTT CTC CT, respectively), together with the 5'-Primer (5'- AAG CAG TGG TAT C AA CGC...
AGA GT). The second round PCR yielded a product of approximately 400 bp, which was cloned into pGEM®-T Easy Vector, and the nucleotide sequences of several clones determined.

**Northern and Southern Blot Analysis** — Total RNA from various tissues was isolated using TRIzol reagent. mRNA was isolated from total RNA using a Dynabeads mRNA Purification Kit. 10 µg of total RNA or 200 ng of mRNA was fractionated on a formaldehyde/agarose gel, transferred to Electra® nylon membrane (Merck, Ltd.) and hybridized with a probe corresponding to the open reading frame of the ecdysone oxidase cDNA. The probe was radiolabeled with [α-32P]-dCTP using a Ready-To-Go™ DNA labeling kit (-dCTP) (Amersham Pharmacia Biotech, Ltd.) and loading was normalized by probing with a mouse 18S ribosomal RNA probe when using total RNA or with a *S. littoralis* muscle actin partial fragment corresponding to the coding region (unpublished data) when using mRNA. Prehybridization and hybridization were carried out using ULTRAhyb™ (Ambion, Inc.) under the conditions recommended by the manufacturer. The blots were washed at low stringency (2 x SSC and 0.1% SDS, at 40°C) and labeled bands visualized by autoradiography.

Genomic DNA was prepared as described above. 10 µg aliquots of DNA were digested with *Bam*HI, *Eco*RI, *Hind*III or *Sal*I, fractionated on a 1% agarose gel, transferred to a nylon membrane, and hybridized using radiolabeled probes corresponding to different regions of ecdysone oxidase cDNA. Hybridization was carried out in 6 x SSC, 5 x Denhardt’s reagent, 0.5% SDS and 100 µg/ml of denatured, fragmented salmon sperm DNA at 55°C overnight. The blot was washed at low stringency (2 x SSC, 0.1% SDS, at 40°C), and labeled bands visualized by autoradiography.

**Baculovirus Expression of Ecdysone Oxidase** — Due to difficulty in amplifying the full length of the coding region, two fragments (115-1634 and 1589-1914) were amplified using RT-PCR separately, which were subsequently digested with *Eco*RV
and ligated. For this PCR step, PLATINUM® Pfx DNA Polymerase (Life Technologies, Ltd.) was used according to the manufacturer’s instructions. This cDNA containing the complete open reading frame of ecdysone oxidase was inserted into pSynXIV VI’X3 vector (24). This construct was cotransfected into Sf21 cells (approximately 2x10^6 cells) with vSynVI’gal DNA (24). Recombination between viral sequences flanking ecdysone oxidase cDNA in pSynXIV VI’X3 and homologous sequences in the viral genome resulted in the replacement of the β-galactosidase in vSynVI’gal with the entire ecdysone oxidase cDNA and a functional polyhedrin gene. The recombinant viruses were identified by screening based on their β-galactosidase negative, occlusion-body positive plaque phenotype. Their structures were confirmed by restriction endonuclease analysis following Southern blotting. Procedures used for maintenance of Sf21 cells, for propagation of vSynVI’gal and for the construction and characterization of recombinant viruses are described in (24).

SDS-PAGE — 2x10^6 High Five™ cells infected at the multiplicity of infection (MOI) of 10 with wild type Autographa californica nucleopolyhedrovirus (AcMNPV), or with recombinant baculovirus, were homogenized at different times post-infection in 1 ml of 100 mM Tris buffer (pH 8.0), containing 0.05% NaN_3. The homogenate was centrifuged at 17,000 x g for 10 min and the supernatant was recentrifuged at 170,000 x g for 1 h to obtain the cytosolic fraction. 5 µl of each fraction was added to an equal volume of SDS sample buffer and boiled for 5 min before loading onto an 8% polyacrylamide gel (22). After electrophoresis, the gel was stained with Coomassie brilliant blue and destained to allow visualization of the protein.

Enzyme Assay — Assay of the recombinant ecdysone oxidase activity was performed in triplicate by modification of the method described in (21). 2x10^6 High Five™ cells infected at MOI of 10 with either wild type or recombinant baculovirus were homogenized at 70 h post-infection in 1 ml of 100 mM Tris buffer (pH 8.0), containing 0.05% NaN_3. The homogenate was centrifuged at 17,000 x g for 10 min, and the
supernatant was recentrifuged at 170,000 x g for 1 h to obtain the cytosol fraction. A 5 µl aliquot was assayed by incubation for 10 min at 40°C in a 50 µl assay mixture consisting of 0.1 M sodium phosphate buffer (pH 6.6), and 60 µM ecdysone. Assays were quenched with 50 µl of methanol and proteins were removed by centrifugation. The supernatant was analyzed by reversed-phase HPLC. Protein concentration was determined by the method of Bradford using a dye-binding assay (Bio-Rad) with bovine serum albumin as a standard. Three independent experiments were performed for each assay, with all assays being carried out in duplicate.

Injection of Insects with 20-Hydroxyecdysone and RH-5992 — Injections were made via dorsal segments, and injection sites were sealed with low melting point wax. RH-5992 or 20-hydroxyecdysone was administered to the larvae in methanol (2 µl) as double injections (1 µg of RH-5992 or 4 µg of 20-hydroxyecdysone per insect) at 42 h and 48 h into the VIth instar. Methanol-injected larvae served as controls.

Kinetic Studies — For the kinetic study of ecdysone oxidase, the reactions were performed as described above, but for 5 min and included 1.85 kBq of [23,24-3H2]ecdysone and various concentrations of unlabeled ecdysone (15).

High-Performance Liquid Chromatography (HPLC) — Ecdysteroids were analyzed by HPLC through a C18 Nova-Pak cartridge (10 cm x 5 mm; Waters Associates) on a Waters instrument (Waters Associates) linked to a 440 ultraviolet detector set at 254 nm, and eluted with an isocratic solvent system consisting of acetonitrile:0.1% (v/v) TFA in water [22:78 (v/v)] at 1 ml/min (21).
RESULTS

Cloning of the Gene Encoding Ecdysone Oxidase — A PCR-based cloning strategy, as detailed in the "Experimental Procedures", allowed us to obtain a cDNA fragment corresponding to the sequence between nucleotides 139 - 434 in Fig. 2. Gene-specific primers derived from this sequence were synthesized and used for 5'-RACE to obtain the 5'-end of the cDNA. 5'-RACE produced a cDNA clone of approx. 390 bp which contains a putative translation start site at position 115.

A genomic library prepared from mixed tissues of *S. littoralis* was screened with the initial 295 bp fragment, resulting in isolation of three clones. Restriction digestion and Southern blot analysis suggested that the three clones were identical. Gene-specific primers derived from the sequence of the genomic clone were synthesized and used for RT-PCR and 3'-RACE. The amplified products were cloned and sequenced. Taken together, all overlapping cDNAs span a total of 2.8 kb. The polyadenylation signal (AATAAAA) is located at position 2838. As shown in Fig. 2, using the first ATG as the start codon, the full-length ecdysone oxidase cDNA encodes a protein of 599 amino acids with a predicted molecular mass of 65206.1, which is consistent with its apparent *M*<sub>r</sub> observed in our SDS-PAGE analysis (21). This conceptually translated protein starts with MCYAVGGGC and is consistent with the NH<sub>2</sub> terminal fragment sequence we determined of XYAVGGX, assuming that the initial methionine has been cleaved as is commonly observed (25). The first Cys residue may have been obscured due to the generally high background in the first sequencing cycle coupled with poor sensitivity of detection of this residue. The protein is mildly acidic with an estimated pI of 5.82.

Genomic Structure of the Ecdysone Oxidase gene — Genomic DNA was prepared from combined fat body and Malpighian tubules and digested with various restriction enzymes, of which *Sal*I and *Bam*HI cleavage sites, are found in the ecdysone oxidase genomic clone, whereas *Eco*RI and *Hind*III sites are not. The resultant DNA fragments
were analyzed by Southern blot using probes representing different regions of the
cDNA. As shown in Fig. 3, two bands were observed in BamHI (approximately 2.6 kb
and 2.3 kb) and SalI (approximately 5 kb and 1 kb) digested samples, when the blot was
subjected to hybridization with a probe representing the whole coding region of
ecdysone oxidase cDNA. However, when the same blot was re-probed with a cDNA
fragment that corresponded to the NH₂-terminal region, essentially only one band was
detected in all samples. Again, a probe that represents the C-terminal region detected
only one band in each case; in the case of EcoRI and HindIII, only a large band was
observed. This analysis is most simply interpreted if ecdysone oxidase is encoded by a
single copy gene in the S. littoralis genome. A weak band of 2.6 kb in the SalI digests
of Figs. 3A and 3C is most probably due to a polymorphic SalI site downstream of the
gene, since this genomic DNA was prepared from 10 individuals.

The genomic sequence of ecdysone oxidase showed that the mRNA was encoded by 5
exons. Exons 3, 4 and 5 contain the coding region (Fig. 4A), and all exon-intron
boundaries followed the ‘GT-AG rule’ for splice donor and acceptor sequences. A
MOTIF (http://motif.genome.ad.jp/) search revealed that the 5′-flanking region of the
transcription initiation site contains the arthropod capsite motif (26) at −1, a putative
TATA box motif at −28, a putative Broad-Complex binding motif (27) at −578 and CF2
binding motifs (28) at −35 and −65 (Fig. 4B). There is a repeat sequence (TTA)_6 at −
285, which is likely to be a microsatellite sequence. However, the 11 bp motif
(CT/G_A/G/AGTAN/G/T) is repeated 5 times at −548, −226, −171, −131 and −107, and
since it resembles the binding motif for FTZ-F1, a transcription factor that belongs to
the nuclear hormone receptor superfamily (29, 30), it is very likely that the ecdysone
oxidase in S. littoralis is regulated by a factor of this type.
Similarity of the Deduced Amino Acid Sequence of Ecdysone Oxidase to Proteins of the GMC Oxidoreductase Family — The deduced amino acid sequence of the coding region showed similarity to enzymes in the GMC oxidoreductase family (Fig. 5), such as *Drosophila melanogaster* glucose dehydrogenase (49%), *Drosophila pseudoobscura* glucose dehydrogenase (49%), *Escherichia coli* choline dehydrogenase (47%), *Rhizobium meliloti* choline dehydrogenase (45%), *Pseudomonas oleovorans* alcohol dehydrogenase (45%), *Aspergillus niger* glucose oxidase (38%) and *Prunus serotina* (R)-mandelonitrile lyase isoform 1 (40%). The ecdysone oxidase amino acid sequence exhibited features characteristic of the proteins of the GMC oxidoreductase family (31, 32), such as the four putative FAD-binding domains (32) corresponding to amino acid residues 49-80, 300-319, 548-570 and 578-598, the flavin attachment loop corresponding to residues 127-144, and the putative active site (32) at His-538, that are highly conserved in members of this superfamily (32).

**Tissue Distribution and Developmental Expression of Ecdysone Oxidase** — As demonstrated in Fig. 6, a cDNA probe representing the protein coding region of ecdysone oxidase detected a transcript (2.8 kb) in midgut prepared from the prepupal stage of the last larval instar. No detectable expression at this stage of development was found in fat body, Malphigian tubules, hemocytes, and epidermis.

The quantitative profile of the specific activity of ecdysone oxidase during the last instar is shown in Fig. 7A. The ecdysone oxidase activity was detected from an early stage of the last larval instar, and it reaches a peak of 75 nmol/h/mg at 103 h, just after a time when the ecdysteroid titer (data from ref. 6) in hemolymph reaches a peak. The activity quickly decreased with the onset of pupation, and increased again during the pupal stage.

Northern blot analysis of mRNA isolated from midgut at different developmental stages of the last larval instar (Fig. 7B) revealed that the ecdysone oxidase mRNA is mainly expressed in the prepupal stage of the instar and in the early pupal stage. The mRNA started to be detectable at 20 h into the last larval instar, although its expression
level during the feeding stage was very low. It started to increase in intensity from 66 h, and reached a peak at 96 h, and quickly decreased just before pupation, rising again after pupation. The developmental profile of ecdysone oxidase mRNA expression slightly preceded that of the enzyme activity. Attempt to normalize the northern blot have proven problematic, because of developmental changes in the transcript levels of the probes and for normalization.

*Induction of the Ecdysone Oxidase Transcript by 20-Hydroxyecdysone and RH-5992* — To examine possible induction of ecdysone oxidase mRNA transcription, we injected larvae twice at 42 h and 48 h into the last larval instar with 20-hydroxyecdysone or RH-5992 i.e. before the natural increase in enzymatic activity. Total RNA was isolated from the midgut of the larvae 4 h or 18 h after the final injection and analyzed by northern blotting. The mRNA encoding ecdysone oxidase was strongly induced by RH-5992 after the final injection (Fig. 8).

*Expression of Ecdysone Oxidase in High Five™ cells* — Monolayers of High Five™ cells were infected with wild type, or with recombinant, baculovirus containing the coding region of ecdysone oxidase, and the cells were collected at intervals between 24 and 70 hours. A polypeptide of approximately 65 kDa, as determined by SDS-PAGE, increased in intensity with time post-infection in samples infected with the recombinant baculovirus, but not in samples infected with the wild type virus (Fig. 9).

The recombinant virus-infected cell lysate was enzymatically functional in oxidizing ecdysone to 3-dehydroecdysone (Fig. 10). As shown in the reversed-phase HPLC UV chromatograms, there was appreciable conversion of ecdysone into 3-dehydroecdysone from ecdysone oxidase recombinant virus-infected cell lysate, with no detectable conversion in the case of wild-type virus-infected cell lysate or uninfected High Five™ cell lysate alone (data not shown). The product of the enzyme reaction was purified by HPLC and analyzed by Liquid Secondary Ion Mass Spectrometry (LSIMS) using glycerol matrix and a Cs⁺ primary ion beam on a VG Quattro triple quadrupole mass
spectrometer (VG Biotech, Altrincham, UK). In the negative ion spectrum, the most prominent ion was at m/z 461 [M-H], consistent with an M, of 462 as expected for 3-dehydroecdysone.

**Kinetic Parameters** — The kinetic parameters, K_m and V_max, of recombinant ecdysone oxidase were investigated. The effect of substrate concentration on the activity of ecdysone oxidase is shown in Fig. 11. A characteristic hyperbolic curve was observed, typical of an enzyme obeying Michaelis-Menten kinetics. The K_m and V_max values determined from the hyperbola (5.10 µM and 0.036 nmol/min/µg protein) and Hanes plot (5.80 µM and 0.038 nmol/min/µg protein) were broadly in agreement.
DISCUSSION

3-Epimerization of ecdysteroids is one of the inactivation pathways of these hormones and ecdysone oxidase converts ecdysteroids into 3-dehydroecdysteroids as intermediates in the formation of 3-epiecdysteroids (Fig. 1). Using a reverse transcription-PCR-based cloning strategy, employing degenerate primers designed on the basis of the partial amino acid sequences of the purified S. littoralis ecdysone oxidase, together with 5'- and 3'-RACE, the complete cDNA encoding the enzyme was isolated and sequenced. The predicted amino acid sequence of the cDNA contained our experimentally determined NH₂-terminal and internal proteolytic peptide sequences of the enzyme. Furthermore, when the cDNA encoding ecdysone oxidase was expressed using a baculovirus expression system, a polypeptide band of approximately 65 kDa was observed on SDS-PAGE that increased in intensity with time of culture (Fig. 9). The lysate from recombinant baculovirus-infected cells showed ecdysone oxidase enzymatic activity. Collectively, these results indicate that the cloned cDNA encodes ecdysone oxidase.

Southern blot analysis indicates that ecdysone oxidase is probably a unique gene in the haploid genome of S. littoralis, consisting of five exons. Exons 3, 4 and 5 contain the coding region (Fig. 4A). Exon-intron boundaries follow the ‘GT-AG rule’ for the splice donor and acceptor sequences. There are motifs corresponding to an arthropod capsite at −1, the putative TATA box at −28 and the CF2 binding motifs at −35 and −65 in the 5′-flanking region (Fig. 4B).

Database searching revealed that ecdysone oxidase belongs to the GMC oxidoreductase superfamily (31, 32). It is clear that the predicted amino acid sequence of this enzyme has significant similarity to other enzymes in this family, and that in particular, four putative FAD-binding regions [amino acid residues 49-80, 300-319, 548-570 and 578-598], (32), the flavin attachment loop [residues 127-144], and His 538, essential for catalytic function, are conserved (32). These data indicate that ecdysone oxidase in S. littoralis is an FAD flavoprotein. It is noteworthy that ecdysone
oxidase has low sequence similarity (27%) to another FAD-dependent enzyme catalyzing 3-oxidation of a sterol, cholesterol oxidase from Brevibacterium sterolicum (33). However, in the case of this substrate, the conformation of the sterol ring structure is quite different from that in ecdysteroids owing to the A/B cis ring junction in the latter. Furthermore, the significance of the observation that the primary sequence alignment shows that ecdysone oxidase has highest similarity to glucose dehydrogenase from Drosophila is unclear. Although ecdysone oxidase activity has been reported in Drosophila (34), no sequence has been annotated as a putative ecdysone oxidase. There is some significant similarity in gene structure between the ecdysone oxidase in S. littoralis and the two Drosophila glucose dehydrogenase genes. All 3 have a large intron close to the transcription start site and upstream of the start codon. The last, relatively small, intron largely separates the first FAD binding domain from the remainder of the coding region, and consequently the last exon is large. However the S. littoralis ecdysone oxidase gene has an additional intron compared to these glucose dehydrogenase genes. The P. serotina (R)-mandelonitrile lyase gene has a different structure.

Ecdysone oxidase activity has been demonstrated in midgut, fat body, hemolymph and integument of larvae in various insect species, although the predominant activity appears to be in midgut during the larval stage (11, 35, 36). Our northern blot analysis shows that the mRNA for ecdysone oxidase was highly expressed in the midgut, but undetectable in other tissues examined at the prepupal stage in S. littoralis under our conditions (Fig. 6). These data indicate that midgut is the main tissue which can produce ecdysone oxidase during the prepupal stage.

The developmental profile of the enzymatic activity (Fig. 7) revealed that the ecdysone oxidase is predominantly expressed during the prepupal stage of the last larval instar. Similarly, northern blot analysis revealed that the expression pattern of the mRNA corresponds closely to the enzyme activity profile. This result suggests that ecdysone oxidase is primarily regulated at the transcriptional level. The fact that high expression of ecdysone oxidase occurs in the late stage of the last larval instar, when
the active molting hormone titer is highest (6), suggests that the enzyme may play a role in deactivation of endogenous ecdysteroids. In *Manduca sexta*, ecdysone oxidase activity also increases at a similar developmental stage as observed in *S. littoralis* (36). Also, the developmental profile showed that ecdysone oxidase is highly expressed in the early pupal stage. In many insects, high ecdysone oxidase activity has been detected in pupae (35), but the significance of this enzyme in the pupal stage is unclear.

In *M. sexta*, it has been shown that ecdysone oxidase activity can be induced by the ecdysteroid agonist, RH-5849, but not by 20-hydroxyecdysone (37). Similarly, in the current work, mRNA for ecdysone oxidase is induced by the ecdysteroid agonist, RH-5992, with no clear induction by 20-hydroxyecdysone (Fig. 8). Furthermore, *in vivo* expression of this enzyme seems to developmentally follow to some extent the ecdysteroid titer determined previously in hemolymph of a different batch of insects (Fig. 7). These data suggest that the gene encoding ecdysone oxidase is ecdysteroid-responsive. Although there is no obvious match to the ecdysone receptor binding motif (38, 39) in the 5'-flanking region of this gene (Fig. 4B), there is an apparent Broad-Complex binding motif (27), which is an ecdysone-responsive element that is known to be required for induction of late genes. Interestingly, there are five copies of a motif in this promoter region, that is similar to the FTZ-F1 binding motif (30). FTZ-F1 is a member of the nuclear hormone receptor superfamily and is expressed in the prepupal stage (40, 41). It is induced by ecdysteroids and functions as a regulatory factor for the late genes (30, 42). Therefore, it is quite possible that ecdysone oxidase is regulated by FTZ-F1 or a similar factor.

Ecdysteroid 26-hydroxylase, another ecdysteroid inactivation enzyme, is also induced by ecdysteroids in *S. littoralis* (43). It is possible that both these inactivation systems may have similar regulatory mechanisms. Both enzymes catalyze the first steps in ecdysteroid inactivation pathways and might be expected to be subject to regulation. By contrast, 3DE 3β-reductase and ecdysone 20-monoxygenase, which are responsible for the activation of ecdysteroids, are not induced by ecdysteroids (Chen, J.-H. unpublished data, 37, 43).
We characterized the kinetics of ecdysone oxidase using the recombinant protein. The results revealed that the $K_m$ for ecdysone of this recombinant enzyme (5 µM) from *S. littoralis* was similar to that (20 µM) previously reported for the partially purified enzyme (15) and was of a similar order of magnitude as those from other species [the blowfly, *C. vicina*, 42 µM (17), 98 µM (18) and *M. sexta*, 13.3 µM (44)].

We previously reported (20) the deduced amino acid sequence of 3DE 3β-reductase from *S. littoralis*, that catalyzes NAD(P)H-dependent reduction of 3DE to yield ecdysone. Although this enzyme catalyzes the reverse conversion to that of ecdysone oxidase, it belongs to the different, Aldo-Keto Reductase superfamily, and there is no significant sequence similarity between them. From this it would seem that these two distinct *S. littoralis* enzymes have evolved independently. A different situation exists in vertebrates where nicotinamide nucleotide cofactor-dependent 3-hydroxysteroid dehydrogenases generally catalyze reversible oxidation-reduction reactions at C-3 of steroids. In fact, oxygen-dependent oxidase-catalyzed oxidation of steroids in vertebrates has not been reported previously, thus, making ecdysone oxidase novel amongst eukaryotic animals. Taken together, these facts suggest that major parts of the system of steroid conversion in insects are evolutionarily distinct from those of vertebrates.
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FOOTNOTE

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The nucleotide sequences reported in this paper have been submitted to the GenBank™ with accession numbers AY035784, AY035785.

†To whom correspondence should be addressed: Cellular Regulation and Signaling Division, School of Biological Sciences, University of Liverpool, Life Sciences Building, Crown Street, Liverpool, L69 7ZB, U.K. Tel: +44 151 794 4352; Fax: +44 151 794 4349; email: reeshh@liv.ac.uk

†The abbreviations used in this work are: E, ecdysone; 3DE, 3-dehydroecdysone; GMC, glucose-methanol-choline; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcriptase-PCR; MOI, the multiplicity of infection; AcMNPV, Autographa californica nucleopolyhedrovirus; LSIMS, liquid secondary ion mass spectrometry.
Figure legends

Fig. 1. Enzymatic interconversions of ecdysone, 3-dehydroecdysone and 3-epiecdysone.

Fig. 2. Nucleotide and deduced amino acid sequences of ecdysone oxidase. The cDNA sequence is indicated on the top line, and the deduced amino acid sequence is on the second line. Intron positions and lengths are indicated above the nucleotide sequence. The putative polyadenylation signal is double-underlined, and the amino acid sequences obtained from the purified ecdysone oxidase are underlined. SalI, BamHI and EcoRV restriction sites are shown in boxes. Numbers on the right refer to the last amino acid residue on each line of the respective protein sequences. Numbers on the left refer to the first residue on each line of the respective nucleotide sequences.

Fig. 3. Southern blot analysis of S. littoralis genomic DNA. 10 µg of genomic DNA was digested with various restriction enzymes as indicated on the top of each panel. The blot was hybridized with 32P-labeled cDNA corresponding to the entire coding region (A), a region from the start codon to the SalI site (115-781, Fig.1) (B), and a region from the BamHI site to the stop codon (959-1914, Fig.1) (C). DNA marker sizes are indicated on the left.

Fig. 4. Genomic organization of the ecdysone oxidase gene. (A) The five exons are represented by boxes, with the coding regions hatched. (B) The putative promoter sequence of the ecdysone oxidase gene. Bold italics indicate transcribed residues (+1 to +20) The TATA box motif is underlined and the arthropod capsites motif is double-underlined. Predicted transcription factor binding motifs are indicated by arrows.

Fig. 5. Alignment of the deduced amino acid sequences of ecdysone oxidase and the most similar database proteins. The deduced ecdysone oxidase sequence (EO Spodoptera) was compared with all sequences in the SWISS-PROT database using the BLAST program. Only the amino acid sequences of the most similar proteins present in
the GMC oxidoreductase family are shown in the alignment. Gaps introduced to optimize alignment are indicated by hyphens. Identical amino acids between ecdysone oxidase and at least one other sequence are indicated in black boxes. Numbers on the right refer to the last amino acid residue on each line of the respective protein sequences. The accession number and the percentage similarity to EO Spodoptera of each protein are as follows: *Drosophila melanogaster* glucose dehydrogenase (GDH), P18173, 49%; *Drosophila pseudoobscura* glucose dehydrogenase (GDH), P18172, 49%; *Escherichia coli* choline dehydrogenase (CHD), P17444, 47%; *Rhizobium meliloti* choline dehydrogenase (CHD), P54223, 45%; *Pseudomonas oleovorans* alcohol dehydrogenase (ADH), Q00593, 45%; *Aspergillus niger* glucose oxidase (GOD), P13006, 38%; *Prunus serotina* (R)-mandelonitrile lyase isoform 1 (LYA), P52706, 40%. The alignment was constructed by use of the CLUSTALW program.

Fig. 6. **Northern-blot analysis of the tissue distribution of ecdysone oxidase.** A 10 µg aliquot of total RNA from various tissues during the prepupal stage of the last larval instar was used. The blot was hybridized with 32P-labeled ecdysone oxidase cDNA probe which corresponded to the whole protein coding region. The same blot was stripped and reprobed with 18S rRNA probe.

Fig. 7. **The developmental profile of ecdysone oxidase in midgut during the sixth instar.** (A) Profile of the enzymatic activity. Boxes on the abscissa refer to the light and dark phases and insects were synchronized at the 5th/6th instar molt occurring over a scotophase. Each point represents the mean of three separate investigations with each assay being carried out in duplicate; bars represent the standard error. Enzymatic activity ( ), ecdysteroid titer in hemolymph ( ), (ecdysone equivalents measured by RIA in a different batch of insects, data from ref. 6). G is gut purge. W is wandering. (B) Northern blot analysis. 200 ng of polyA+ RNA from midgut at various time points within the sixth larval instar as indicated was used. The blots were hybridized with a 32P-labeled ecdysone oxidase cDNA probe corresponding to the open reading frame.

Fig. 8. **Effect of 20-hydroxyecdysone and agonist on the induction of ecdysone oxidase mRNA.** Last instar larvae were injected with methanol, 2 µg of 20-
hydroxyecdysone or 1 µg of RH-5992 at 42 h and 48 h into the VIth instar, and dissected at the times indicated. 200 ng of polyA+ RNA was analysed by northern blotting using 32p-labeled ecdysone oxidase (coding region) or actin cDNA as probes.

Fig. 9. SDS-PAGE analysis of protein synthesis in High Five™ cells infected with recombinant or wild-type baculovirus. Infected cells were lysed and collected at 24, 41, 48 and 70 h post-infection. Each extract was analyzed by SDS-PAGE and visualised by Coomassie Brilliant Blue (see Experimental Procedures). The positions of protein markers are shown on the left. The position of ecdysone oxidase is shown on the right.

Fig. 10. Enzymatic activity of recombinant ecdysone oxidase. Cytosol fractions of High Five™ cells infected with wild-type or recombinant baculovirus were prepared, incubated with ecdysone, and the products were analysed by reversed-phase HPLC (see Experimental Procedures). The positions of elution of authentic ecdysone (E), 3-epiecdysone (E’) and 3-dehydroecdysone (3DE) are shown.

Fig. 11. Effect of various concentrations of ecdysone on the velocity of ecdysone oxidase. Recombinant ecdysone oxidase (cytosolic fraction) was incubated under standard enzyme activity assay conditions (see Experimental Procedures). Each assay was performed in duplicate and the data presented are means for three independent experiments. The insert shows the Hanes plot of ecdysone oxidase activity.
(A)

Exon 1

(B)

GATCGTTGGGATTCAGGGATTGGGGAGATTTGGGGAAAGGGGGAGG GTAATTAGGCTTCTGG -629

TAACGTCAAGTCACACGCAGAAGCAAACACAAGAAGCTTTGCT -569

TTTATTTAATGCTTCATGCAACAGAGTGTACAAAGAAAGGTTGGGTTTAATGCAGGTAGCAT -509

Broad-Complex

TTTCATTACCAGTCTGACCTTTGGGTAGGGCCGGTGATTATCCTCACTCCGTCAGGCACGGCCACTT -449

CCTGCCGAAGCAGCATGGCTCTCCACCTCTTGAAATCTTTTCTCTTAGTTCTCCATATCGATGG -379

GTTACCCTGTATCCTTTGTAGCGTTAAGTTTTCAATTGTATACGGTGACGCCGCCACCTTCACTTATTATTATTATTATT -329

GGTATTGATGGTTCAACCAGGAGCTAAGGAGATTTTTGGGGCTTTATTATTTATTT -269

ATAATATGAAAATGTCGACTTTGTTATCTGCATCGATTTTATGACTCACAGGTATTTCAGGAAA -209

AAAACAAGACAATGTCAAAGTGGAGTAAAGTGCTCTCAGACTGTTATCGACTTTT -149

TCAGTTGGAGTGTGCTGACATAAAGGGTTATGTTAAACCTGAGTAATAATCTGGATGGCC -89

TGATTATGTCACCTGATACGCCTCATATATACATGTACCTCTAGCCGGCTCCCATAC -29

ATATAGCTGCACGCCCGCGCGAGTTGCTCAGCTACCCCGCAGCAGTTATTG

CF2

CF2

CF2
(A) Specific Activity (nmol/h/mg protein) vs Developmental Time (hours)

| Developmental Stage | Specific Activity (nmol/h/mg protein) |
|---------------------|---------------------------------------|
| Feeding             | 0                                     |
| G/W                 | 20                                    |
| Prepupa             | 48                                    |
| Pupa                | 72                                    |

(B) VI\textsuperscript{th} instar vs Pupa

| VI\textsuperscript{th} instar | Pupa |
|-------------------------------|------|
| (kb)                          | (hours) |
| 20, 25, 43, 48, 66, 72, 90   | 96, 100, 114 |
| 0, 18, 24                     | 0, 18, 24 |

Ecdysone oxidase
4 h
Methanol  20E  RH5992
18 h
Methanol  20E  RH5992

Ecdysone oxidase

(kb)
4.40
2.37

Actin
Regulation of Ecdysteroid signaling: Cloning and characterization of Ecdysone oxidase, a novel steroid oxidase from the cotton leafworm, Spodoptera littoralis
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