Regulation of ecdysteroid signalling during *Drosophila* development: identification, characterization and modelling of ecdysone oxidase, an enzyme involved in control of ligand concentration

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The steroidal moulting hormones (ecdysteroids) mediate developmental transitions in insects, and their regulation is mainly controlled by the production and inactivation of these steroid hormones at the appropriate developmental times. One route of metabolism of ecdysteroids in insects involves EO (ecdysone oxidase)-catalysed conversion into 3-dehydroecdysteroid, which undergoes reduction to the corresponding 3-epiecdysteroid. By a twin-stranded bioinformatics approach, employing both phylogenomics and model structure-based analysis, we first predicted that DmEO (the EO of *Drosophila melanogaster*) corresponds to the protein product of gene CG9504. When CG9504 was expressed in COS7 cells, significant conversion of ecdysone into 3-dehydroecdysone was observed. Quantitative PCR and enzyme assay showed that DmEO was mainly expressed in the midgut during the late instars at a time corresponding to a hormone titre peak. DmEO shares only 27 % amino acid sequence identity with *Spodoptera littoralis* (Lepidoptera) EO, yet key substrate-binding residues are well conserved. A model of DmEO is consistent with an inability to catalyse reaction of cholesterol derivatives. The significance of DmEO in ligand activation is discussed in relation to new evidence suggesting that 3-dehydro- and 3-epi-ecdysteroids may be functionally active as ligands in a novel, atypical ecdysteroid signalling pathway involving the *Drosophila* orphan nuclear receptor, DHR38, rather than being merely hormone inactivation products.

Key words: 3-dehydroecdysone, 3-dehydro-20-hydroxyecdysone, ecdysone receptor, glucose-methanol-choline oxidoreductase (GMC oxidoreductase), metamorphosis.

INTRODUCTION

Moulting, development and aspects of reproduction in insects are controlled by the insect moulting hormones (ecdysteroids) [1]. According to the classical scheme, at specific times in immature stages, the prothoracic glands synthesize and secrete ecdysone, which undergoes 20-hydroxylation in several peripheral tissues, yielding the much more active hormone, 20E (20-hydroxyecdysone) [2]. The ecdysteroid titre exhibits distinct peaks at specific stages in development. In immature stages, these arise by increased ecdysteroid synthesis in the prothoracic glands; however, in *Drosophila* pupae, the source appears not to be these glands [3]. Decreases in titre result from enhanced ecdysteroid inactivation reactions, together with possible elevated excretion. A number of transformations contribute to the inactivation of ecdysteroids, including the formation of 3-epi-(3α-hydroxy) derivatives, which are regarded as hormonally inactive [2,4–6]. Formation of 3-epi-ecdysteroids, which occurs in many insect orders, including both Lepidoptera and Diptera, entails conversion of ecdysteroid into 3-dehydroecdysteroid, followed by nicotinamide nucleotide-cofactor-dependent irreversible reduction to 3-epiecdysteroid (Figure 1) [2,5–10]. These reactions occur with both ecdysone and 20E. EO (ecdysone oxidase; EC 1.1.3.16) is the enzyme that catalyses the oxidation of ecdysteroid and was first demonstrated, characterized and extensively purified from the blowfly, *Calliphora vicina* [11,12].

As a part of our studies aimed at elucidating the regulation of ecdysteroid titre, including the reactions involved in ecdysteroid inactivation, we have characterized EO [10], 3DE (3-dehydroecdysone) 3β-reductase [13] and 3DE 3α-reductase [9] in the cotton leafworm, *Spodoptera littoralis* (Lepidoptera). EO of *S. littoralis* (SlEO) exhibits peak activity late in the last larval instar, just after the maximum hormone concentration, and thus could be involved in ecdysone inactivation. The enzyme has been purified and its cDNA cloned, with conceptual translation and amino acid sequence analysis, suggesting that it is an FAD flavoprotein which belongs to the GMC (glucose-methanol-choline) oxidoreductase family [10]. Furthermore, Northern blot analysis showed that the EO mRNA transcript is mainly expressed in the midgut, and that fluctuation in the expression during development accounts for the change in the enzyme activity during the instar. Induction by RH-5992, an ecdysone agonist, suggests that SlEO is an ecdysone-responsive gene, whose promoter contains several putative binding motifs for the products of the ecdysone-responsive 'early genes', Broad-Complex and FTZ-F1 (Fushi Tarazu-factor 1) [10].

We here report the identification and molecular characterization of EO in the model organism *Drosophila melanogaster* (DmEO). Although the predicted amino acid sequence of DmEO has a low degree of overall sequence identity with that of SlEO, the amino acid residues predicted to bind substrate are well conserved between the two species. Analysis of expression profiles revealed

Abbreviations used: 3DE, 3-dehydroecdysone; 3D20E, 3-dehydro-20-hydroxyecdysone; 20E, 20-hydroxyecdysone; *ecd-1*, *ecdysoneless-1*; EcR, ecdysone receptor; (Dm/Sl)EO, (*Drosophila melanogaster*/*Spodoptera littoralis*) ecdysone oxidase; GMC, glucose-methanol-choline; PDB, Protein Data Bank; RP49, ribosomal protein 49.

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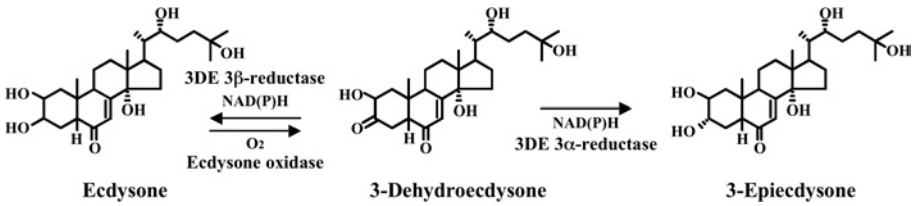

Figure 1 Enzymic interconversions of ecdysone, 3DE and 3-epiecdysone

that the mechanisms of transcriptional regulation of the two enzymes are also very similar.

EXPERIMENTAL

Drosophila strains

Canton S wild-type flies were maintained at 25 °C. Larvae were staged by placing either first or second instar larvae in Petri dishes supplemented with yeast paste and maintaining them at 25 °C. The plates were examined 4 h later for larvae that had moulted. Newly moulted larvae were collected in small vials (Blades Biological, Cowden, Edenbridge, Kent, U.K.) with diet. They were subsequently harvested at 4 h intervals and frozen at − 80 °C. Pre-pupae were staged relative to puparium formation, and aged at 4 h intervals for up to 12 h after puparium formation.

Bioinformatics

D. melanogaster homologues of SlEO [10] were sought in FlyBase [14] using BLAST [15] and the resulting sequence set was aligned using T-COFFEE [16] and manipulated further with Jalview [17]. Sequence-clustering analyses were performed using the programs SEQBOOT, PROTDIST, PROTPARS, NEIGHBOR and CONSENSE of the PHYLIP package [18]. Suitable templates for model building of the EOs were sought by a FASTA search among sequences of the Protein Data Bank (PDB) [19] at the European Bioinformatics Institute (http://www.ebi.ac.uk/fasta33).

Modelling of residues 24–599 of SlEO and of residues 66–583 of DmEO was performed with MODELLER 6 [20] using the glucose oxidase structures [21] of *Aspergillus niger* (PDB code 1cf3) and *Penicillium amagasakiense* (PDB code 1gpe) as templates. No suitable templates for the first 23 residues of SlEO, the first 65 residues of DmEO or the last six residues of DmEO were available. Since the quaternary structure of the GMC class is invariably a dimer, model dimers of the EOs were constructed, with additional MODELLER restraints applied to maintain identical conformation in the two subunits. In the final alignment (Figure 2), SlEO and DmEO shared 22 % and 19–21 % sequence identity, respectively, with the templates. Default regimes of model refinement by energy minimization and simulated annealing were employed. Because of the low sequence similarity between target and template, a rigorous iterative modelling protocol was adopted in which 10 models were constructed and analysed for each alignment variant. These models were analysed for packing and solvent-exposure characteristics using PROSA II [22] and for stereochemical properties using PROCHECK [23]. Possible misalignments were highlighted by positive PROSA II peaks, and variations in alignment of these regions examined. When no further improvements could be achieved, the model with the highest PROSA II score was taken as the final model. The PROSA II score of the final model was used to calculate a pG value [24] in order to provide an estimate of model reliability. Protein structures were superimposed using LSQMAN [25] and visualized using O [26]. The structure of ecdysone was obtained by editing of the crystal structure of 20E [27]. Its structure and that of FAD were added to the final SlEO and DmEO models using the predicted Michaelis complex structure [28] of the distantly related cholesterol oxidase [29] as a guide. Diagrammatic representations of the structures were generated using PyMOL (http://www.pymol.org/). Secondary structures were defined using STRIDE [31].

RNA isolation

Each collection of animals (30 third-instar larvae or prepupae) was homogenized in 200 μl of TRIzol® reagent (Invitrogen). The total RNA was extracted from the homogenate. Each RNA sample was digested by DNase I to eliminate contaminated genomic DNA, and re-purified using the RNeasy Mini Kit (Qiagen).

Quantitative real-time PCR

Real-time PCR was performed with the Opticon 2 thermal cycler (MJ Research, Watertown, MA, U.S.A.) and using the fluorescence dye SYBR® Green I. Total RNA was isolated using RNeasy columns (Qiagen), followed by DNase I treatment using amplification grade DNase-I (Invitrogen) to remove potential contaminating genomic DNA. A sample (2 μg) of DNase-I-treated RNA was used as template to synthesize cDNA using SuperScript II reverse transcriptase (Invitrogen).

Real-time PCR conditions consisted of an initial denaturing step at 95 °C for 10 min, followed by 35 cycles of denaturation for 10 s at 94 °C, annealing for 20 s at 60 °C, extension for 15 s at 72 °C, and 75 °C for 1 s to 'melt off' any primer-dimer before fluorescence readings were taken. To ensure only the fluorescence signal of the product of interest was detected for c(t) (cycle threshold) calculations, melting curve analysis was performed after each PCR from 65°–95 °C, increasing in 0.2 °C increments, and held for 1 s at each step. DmEO-specific primers used were 5′-CCG ATT CCG ATG ACT ACT GG-3′ (forward) and 5′-CGC TGG CAA TTC CGG CAT AA-3′ (reverse). *Drosophila* RP49 (ribosomal protein 49)-specific primers used were 5′-CTC ATG CAG AAC CGC GTT TA-3′ (forward) and 5′-ACA AAT GTG TAT TCC GAC CA-3′ (reverse).

Absolute quantification was performed using gene-specific standard curves for each gene. cDNA encoding the open reading frame of DmEO was cloned into the pSI expression vector (Promega). cDNA encoding RP49 was amplified using the gene-specific primers F and R (5′-TGT CCT CAG CTT CAA GAT-3′ and 5′-ATG TTA TCA ATG GTG CTG CTA-3′ respectively), and this was cloned into the pGEM®-T easy vector (Promega). These plasmids were purified and used as templates to construct standard curves ranging from 10^3–10^9 copies. All reactions were performed in triplicate.

Expression of *Drosophila* genes in COS7 cells

The cDNA sequences of 15 candidate genes containing the entire open reading frame was amplified by PCR and cloned into the

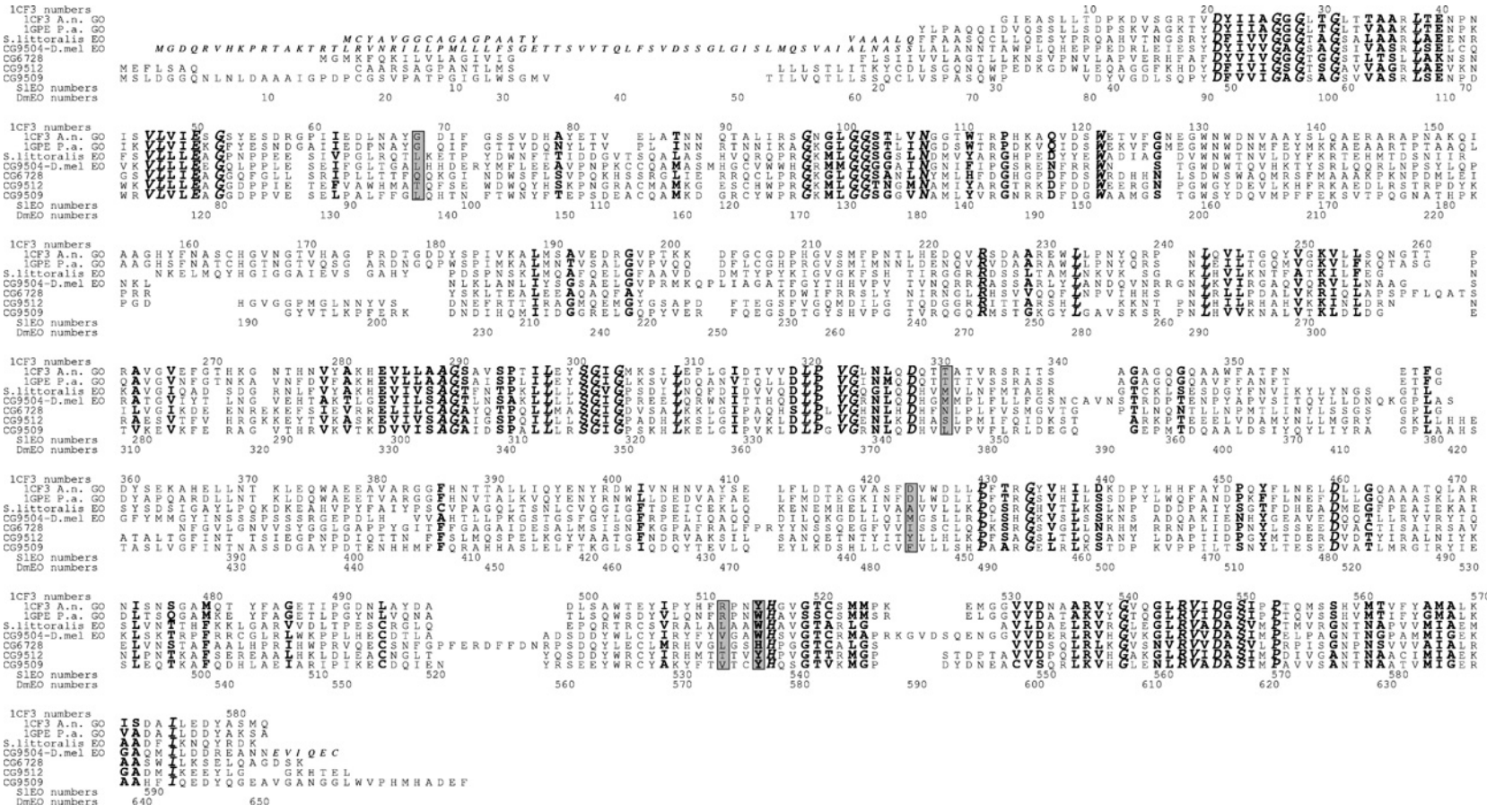

Figure 2 Alignment of SIEO and selected *Drosophila* homologues

Sequence alignment of SIEO and the eventual DmEO with the two glucose oxidase templates (above with PDB codes) and the other three most closely related *D. melanogaster* homologues from the GMC oxidoreductase family as identified by sequence clustering (see Figure 3). Conserved positions are shown in bold and identities additionally shown in italics. Positions predicted to be important for binding of the steroid ring of substrate ecdysone are boxed. Terminal regions of S1EO and DmEO that could not be modelled are shown in italics.

pSI expression vector. COS7 cells were transfected with the expression vector with or without the cDNA by using Lipofectamine™ reagent (Invitrogen). The transfected cells were homogenized at 48 h post-transfection in 50 μl of 20 mM Tris/HCl, pH 8.0. A 20 μl aliquot of this extract was used for enzyme assays.

EO assay

To assay EO activity during *Drosophila* development, whole bodies from 15 larvae, pre-pupa or pupa were homogenized in 20 mM Tris/HCl, pH 7.0. The homogenate was centrifuged at 17 000 ***g*** for 10 min. The supernatant was assayed by incubation for 3 h at 37 °C in 50 μl of assay mixture consisting of 0.1 M sodium phosphate buffer, pH 6.6, and [^{3}H]ecdysone (18.5 kBq; 1.85 TBq/mmol). Assays were quenched with 50 μl of methanol, and proteins were removed by centrifugation. Ecdysteroids in the supernatant were analysed by HPLC using a C_{18} Nova-Pak cartridge (10 cm × 5 mm; Waters Associates) on a Waters instrument (Waters Associates) linked to a 440 UV detector set at 254 nm, and eluted with an isocratic solvent system consisting of acetonitrile/0.1 % (v/v) trifluoroacetic acid in water (22:78, v/v) at 1 ml/min. Three independent experiments were performed for each assay, with all assays performed in duplicate.

In a similar manner, to assay the recombinant EO activity, conversion of [^{3}H]ecdysone into [^{3}H]3DE was measured 2 days after the transfection of COS7 cells with the candidate gene expression construct. The transfected cells (approx. 2×10^6 cells) were homogenized in 100 μl of 20 mM Tris/HCl, pH 8.0. The homogenates were centrifuged at 17 000 ***g*** for 10 min. The supernatant was assayed as above, except that incubation was for only 1 h at 37 °C.

Feeding of insects with RH-0345

Synchronous larvae of the temperature-sensitive mutant *ecd-1* (*ecdysoneless-1*), which shows reduced ecdysteroid synthesis and metabolism [32], were allowed to develop at 20 °C until the beginning of the third larval stage, when they were transferred and maintained at 29 °C for 72 h, after which they were subjected to hormone treatments by feeding. Groups of 30 larvae were subjected to RH-0345 (5 mg/ml diet), an ecdysone agonist [33], or none (control) supplemented food. After 3 h at 29 °C, total RNA was extracted and analysed.

RESULTS

Bioinformatic prediction of the *D. melanogaster* homologue of SlEO

A BLAST search showed that 15 sequences in the *D. melanogaster* genome bore clear similarity to SlEO. The e-values associated with the hits ranged from $2e^{-75}$ to $7e^{-38}$. Seven of the hits had e-values of less than e^{-70}, showing that there was no single clear EO candidate in *D. melanogaster*. When aligned, all 15 *D. melanogaster* sequences shared pairwise sequence identities with SlEO in the range 26–32 %. Mindful of the fact that the top BLAST hit is by no means always the nearest neighbour in evolutionary terms [34], and that sequence clustering is increasingly recognized as an important aid to functional annotation (the 'phylogenomic' approach; [35]), relationships between the *D. melanogaster* sequences and SlEO were explored. As shown in Figure 3, the 15 candidate *D. melanogaster* sequences cluster reliably into three groups. The bootstrapping value associated with this division is 94 for 100 replicates in the tree derived from distance matrix analysis, and 90 for the equivalent tree obtained using maximum parsimony analysis (results not shown). SlEO localizes within the branch also containing the *D. melanogaster* sequences CG6728, CG9504, CG9509 and CG9512. This analysis

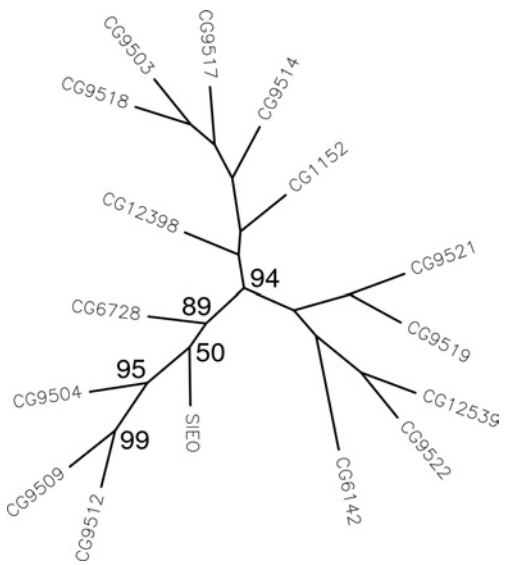

Figure 3 Sequence clustering tree of SlEO and its possible *D. melanogaster* homologues

The tree was calculated from distance matrix data using the PHYLIP package [18]. Selected bootstrapping confidence values from analysis of 100 replicates are indicated.

appeared to narrow the search for the DmEO down to four candidates, but further inference was unreliable, since the structures of the SlEO-containing branches of the distance matrix and maximum parsimony trees differed (results not shown).

As recognized by the current wave of structural genomics projects, knowledge of protein structure is an important aid to function prediction [36]. This approach extends to carefully constructed model structures (e.g. [37,38]). We therefore examined whether a model of SlEO could yield insights into the determinants of ecdysone specificity that would enable a prediction to be made of which *D. melanogaster* gene sequence encoded the EO. To this end, a model of SlEO was constructed using a rigorous, iterative modelling procedure. The templates were chosen based on the results of a FASTA search of the sequences of known structures deposited in the PDB. The two top hits were glucose oxidases from two yeasts, *P. amagasakiense* and *A. niger* [21], which scored e-values of $7e^{-13}$ and $4e^{-11}$ respectively. The third ranking hit was almond hydroxynitrile lyase, with an e-value of $7e^{-8}$, followed by *Phanerochaete chrysosporium* cellobiose dehydrogenase, with e-value $1e^{-5}$. On the basis of these results, only the glucose oxidases were deemed worthy of use as templates. Interestingly, bacterial cholesterol oxidases, also of known structure, were absent from the FASTA results, showing that substrate similarity is not a useful guide to evolutionary relatedness in the diverse GMC oxidoreductase superfamily [39]. Initial models contained regions with unfavourable positive PROSA II profiles [22], several of which could be improved through local alignment changes. The monomer of the final model scored −8.32 by PROSA II analysis, corresponding to a perfect pG value [24] of 1.0 and indicative of a largely accurate target-template alignment. The final model also has good stereochemistry; 88 % of residues are in the core regions of the Ramachandran plot, and none are in disallowed regions.

A molecule of ecdysone (modelled on the crystal structure of 20E [27]) was docked into the final SlEO model using as a guide the predicted Michaelis complex [28] of cholesterol oxidase [29]. Although this is a distant SlEO relative in the GMC oxidoreductase superfamily (see above), the plausibility of the resultant bound

Table 1 The profile of candidate genes of DmEO

All data are derived from Flybase [14]. M, midgut; E, epidermis with attached muscle; S, salivary gland; W, wing disc; C, central nervous system; BPF, before puparium formation; APF, after puparium formation; +, level of expression.

				Developmental expression in midgut		
Name	Cytologic band	Putative function	Highly expressed tissue	18h BPF	2h APF	8h APF
CG9504	X, 13A1	Unknown	M	+++	–	+++
CG9509	X, 13A1	Choline dehydrogenase	M, E	+++	+	+
CG9512	X, 13A1	Choline dehydrogenase	E, M	++	+	+
CG6728	3R, 86E5	Unknown	M, S, E, W, C	++	++	++
CG12398	X, 13A1	Glucose dehydrogenase	M, S, E, W, C	++	++	++
CG9518	X, 12F6-7	Choline dehydrogenase	M, W	++	+	++
CG9503	X, 13A1	Unknown	W	+	++	+++
CG9517	X, 13A1	Glucose dehydrogenase	M, E, W	++	+	++
CG9514	X, 13A1	Choline dehydrogenase	M, E	+	+	+
CG1152	3R, 84D3	Glucose dehydrogenase	E	++	+	+
CG9521	X, 12F6	Choline dehydrogenase	M, W	+	++	+
CG9519	X, 12F6	Choline dehydrogenase	M, E	+	+	+
CG12539	X, 12F5	Glucose dehydrogenase	E	–	+++	–
CG9522	X, 12F5	Choline dehydrogenase	M, W	++	+	+
CG6142	3R, 97A1	Unknown	C, W	++	+	++

conformation was confirmed by an absence of significant steric clashes and the observation that the hydrophobic steroid ring was favourably accommodated in a largely hydrophobic pocket. While the rigidity of the steroid ring facilitated its placement, it was apparent that diverse conformations for the flexible side chain were possible. We therefore confined our predictions of potential contributors to ecdysone specificity to residues neighbouring the steroid ring. These were Leu[96], Met[346], Ala[446], Leu[534] and Trp[537] (shown boxed in Figure 2). The first four of these are different in the glucose oxidases, being represented respectively by the more hydrophilic residues tyrosine, threonine, aspartate and arginine, only the last being reasonably conserved in both glucose and EOs. As predicted ecdysone-binding residues, the expectation was that the *D. melanogaster* sequence encoding EO would contain similar residues to SlEO at these positions. Among the four *D. melanogaster* sequences in the branch of the sequence-clustering tree also containing SlEO, the analysis highlighted CG9504 in which the five positions were occupied respectively by Leu[138], Met[377], Met[484], Val[574] and Trp[577]. The other *D. melanogaster* sequences conserved one or more of these positions, but contained a number of significantly different residues. Thus CG6728 has glutamine, asparagine and histidine at the first, second and fifth positions, and CG9512 has threonine, serine, tyrosine and threonine at the first, second, third and fourth positions. The CG9509 residues conform more closely to the expected pattern: leucine, leucine, phenylalanine, valine and tyrosine. We therefore made the prediction that CG9504 was most likely to represent the DmEO, with CG9509 as a potential alternative candidate. Of the initial set of 15 DmEO candidates identified by sequence-clustering analysis, only CG9504, CG9509 and three more contained hydrophobic residues at all five positions. Interestingly, Flybase [14] suggests a choline dehydrogenase activity for the products of seven genes, a glucose dehydrogenase activity for those of four genes and an unknown activity for those of four genes including CG9504 (Table 1).

Expression of recombinant CG9504 protein in COS7 cells

Monolayers of COS7 cells were transiently transfected with the expression vector containing the coding region of CG9504, and the cells were collected at 48 h after transfection. The CG9504-transfected cell lysate was enzymically functional in oxidizing ecdysone to 3DE (Figure 4). As shown in the reversed-phase HPLC radiochromatograms, there was appreciable conversion of ecdysone into 3DE from CG9504-expressed cell lysate, with no detectable conversion in the case of control cell lysate or untransfected COS7 cell lysate alone (results not shown). Similar expression, in turn, of the other 14 DmEO candidate genes in COS7 cells did not yield EO activity (results not shown).

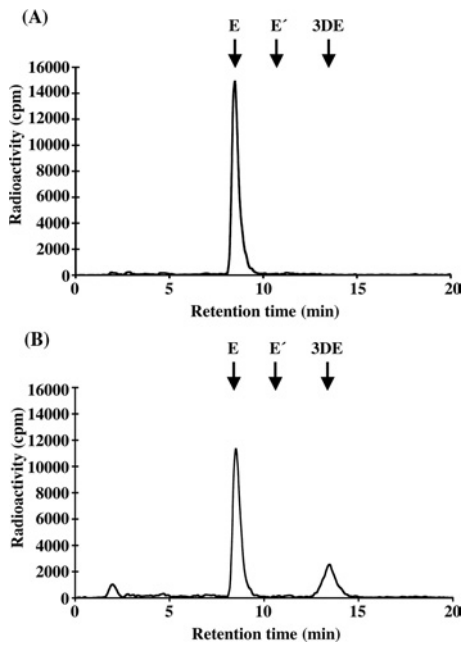

Figure 4 Enzymic activity of recombinant DmEO

Cell extracts of COS7 cells transfected with the expression vector (**A**) or with the expression vector containing the CG9504 cDNA (**B**) were prepared, incubated with [^{3}H]ecdysone, and the products were analysed by reversed-phase HPLC (see the Experimental section). The positions of elution of authentic ecdysone (E), 3-epiecdysone (E′) and 3DE are shown.

Analysis of a model of DmEO

With the identity of CG9504 as an EO established, it too was modelled by the same rigorous approach as applied to SlEO. Once

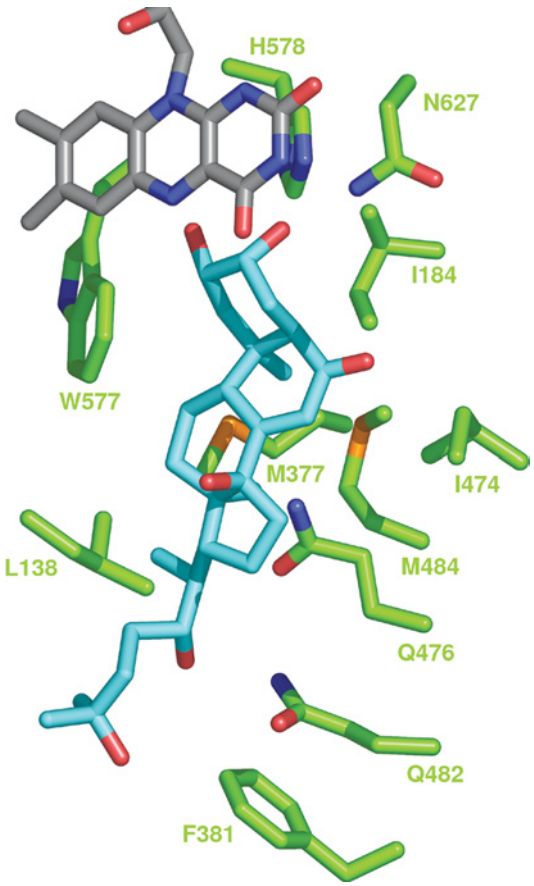

Figure 5 Predicted substrate-binding mode of DmEO

Residues lining the predicted ecdysone-binding pocket are shown and labelled in green. Ecdysone is drawn in cyan, whereas FAD is drawn in grey. The Figure was made with PyMOL (http://www.pymol.org/).

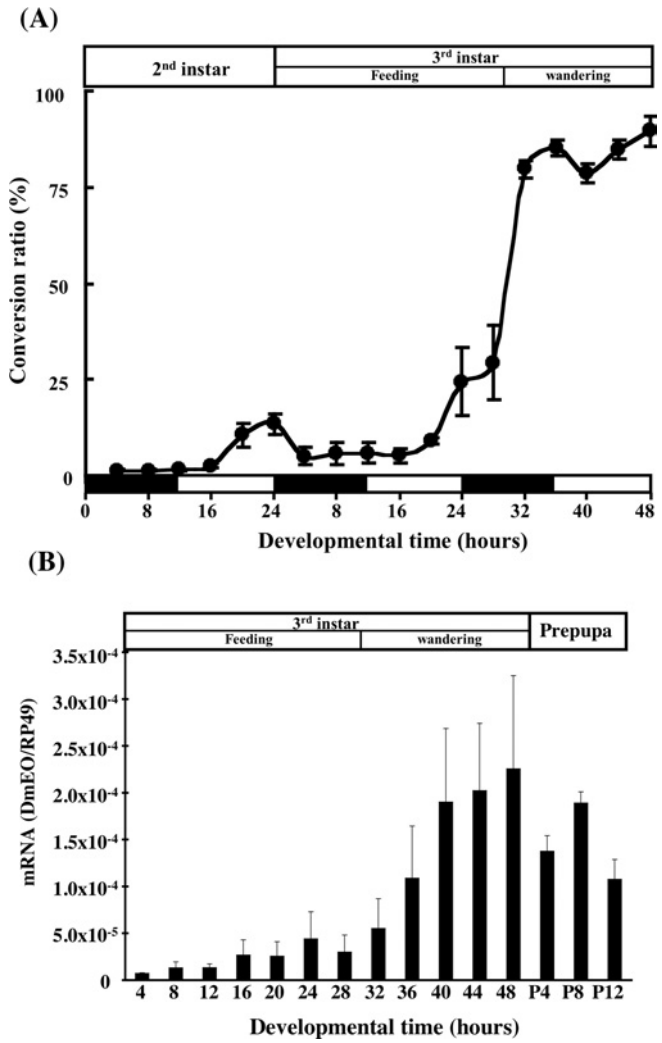

Figure 6 The developmental profile of DmEO

(**A**) Profile of the enzymic activity. Boxes on the abscissa refer to the light and dark phases. Each point represents the mean of three separate investigations with each assay being carried out in duplicate; bars represent ± S.E.M. (**B**) Profile of the mRNA expression. The mRNA level was measured by quantitative PCR, as described in the Experimental section. Results are means ± S.E.M. with three independent samples for each time point. P4, P8 and P12, 4, 8 and 12 h from the start of pupariation respectively.

again the final model performs well by protein structure validation measures. The DmEO monomer scores −7.46 by PROSA II analysis (corresponding to a near-optimal pG value of 0.98), has no residues in disallowed regions of the Ramachandran plot and 88 % in the most favoured regions. The resulting predicted binding mode for substrate shows excellent steric and chemical complementarity with the protein model (Figure 5). Binding residues are mainly hydrophobic, with the exceptions of Gln^{476} and Gln^{482}, each of which is suitably placed to hydrogen-bond to hydroxy groups (O-14 and O-22 respectively) of the substrate.

As yet, the only experimental characterization of substrate specificity of EOs used blowfly enzyme [12]. Although it would be unjustified to assume that all details of the blowfly enzyme specificity apply to the *Drosophila* enzyme, the absolute inability of blowfly EO to catalyse reaction of cholesterol and some derivatives [12] would be expected to be shared by DmEO. The principal difference between cholesterol derivatives and EO substrates lies in the relationship of the ring bearing the reactive hydroxy group with the other rings. In cholesterol, all rings are approximately co-planar, whereas ecdysone and similar compounds have a pronounced *cis*-fusion between the hydroxy-group-bearing ring A and the others (Figure 5). The DmEO model is consistent with an inability to catalyse reaction of cholesterols since, with the other three rings gripped by residues Leu^{138}, Met^{377}, Gln^{476} and Met^{484}, a co-planar ring, as in cholesterol, would not be placed relative to the FAD in such a way as to permit reaction.

Tissue distribution and developmental expression of DmEO

Quantitative PCR showed that mRNA transcript for DmEO was highly expressed in midgut prepared from the third instar larvae. Very low expression of mRNA (approx. 10 % that in midgut) was detected in fat body and carcass, which includes epidermis, muscles, tracheae and peripheral nerves (results not shown).

The profile of the enzyme activity of EO during the larval stage is shown in Figure 6(A). The enzyme activity was detected from an early stage of second instar, and the rise in activity at the end of the instar is well correlated with the rise in ecdysteroid titre which causes moulting [40]. The activity decreased after ecdysis to the third instar. The enzyme activity starts to increase at 24 h into the third instar, again with a rapid rise at the onset of wandering, corresponding to the presumed small ecdysteroid peak that initiates wandering [40]. Enzymic activity more or less remains at a plateau level during the pupariation peak in haemolymph ecdysteroid titre that begins approx. 6 h before pupariation and peaks at pupariation (48 h) [40].

Quantitative PCR analysis of mRNA isolated from the whole body at different developmental stages of the last larval instar (Figure 6B) revealed that DmEO mRNA is highly expressed in the late stage of the last larval instar and the pre-pupal stage. The mRNA started to be detected at 4 h into the last larval instar, although its expression level during the feeding stage was very low. It started to increase in intensity from 16 h, reached a peak at 48 h, quickly decreased just before puparium formation, and rose again after pupariation. The developmental profile of EO mRNA expression slightly preceded that of the enzyme activity.

Induction of the EO transcript by RH-0345

To examine possible induction of DmEO mRNA transcription, we treated larvae of the *ecd-1* mutant, which cannot produce ecdysteroids [32], with RH-0345, an ecdysone agonist [33]. Total RNA was isolated from the larvae 3 h after treatment and analysed by quantitative PCR. The mRNA encoding DmEO was clearly induced by RH-0345 (approx. 3.2-fold; results not shown).

EO enzymes in other insects of known genome sequence

Candidate EO sequences were sought in the complete genomes of *Anopheles gambiae* [41] and *Apis mellifera* (http://www.genome.gov/11008252) using a similar combination of phylogenomics and analysis of key residues identified by modelling. BLAST initially identified 27 candidates in mosquito and 16 in bee. The five key positions shown in Figure 2 are occupied respectively, starting at the N-terminus, by Leu, Met, Ala, Leu, Trp in SlEO and by Leu, Met, Met, Val, Trp in DmEO. Among the mosquito candidates, SNAP00000008130 (SNAP identifiers are those used by the Ensembl annotation project at http://www.ensembl.org) had the closest match, with Leu, Met, Phe, Leu, Tyr. Two further candidates were of sequence Leu, Ala, Phe, Phe, Tyr and Leu, Ala, Phe, Tyr, Tyr. Significantly, SNAP00000008130, but neither of the two further candidates, was contained within the minimal sequence clustering sub-tree, as calculated by protein distance matrix analysis, that contained both DmEO and SlEO. SNAP00000008130 was also located much closer to DmEO and SlEO than the two further candidates in a maximum parsimony tree, and therefore represents a strong candidate for the *A. gambiae* EO.

Only seven of the 16 bee candidate sequences contain no hydrophilic residue within the five key positions, and four of these seven are apparently incomplete in the current set of predicted peptides encoded by the *A. mellifera* genome. Since none of the complete sequences are grouped with known EOs in the sequence-clustering trees, nor do they possess a set of key residues similar to those in DmEO and SlEO, identification of EO candidates in *A. mellifera* should await a later genome draft with more accurately predicted encoded peptides.

DISCUSSION

3-Epimerization of ecdysteroids is one of the major metabolizing pathways of these hormones, and EO converts ecdysteroids into 3-dehydroecdysteroids as intermediates in the formation of 3-epiecdysteroids (Figure 1). Even with the SlEO sequence available, the species-hopping to *D. melanogaster* proved non-trivial. A straightforward BLAST search with SlEO in the *D. melanogaster* genome produced no fewer than 15 candidates. Two powerful and complementary bioinformatics approaches, phylogenomics and model structure analysis, were therefore employed. This twin-stranded bioinformatics strategy reduced the number of candidates first to four, and then to one favoured candidate, CG9504, which, through expression in COS7 cells, was shown to encode DmEO. Remarkably, CG9504 occupied last place in the BLAST-derived list of 15 candidates. Although DmEO (CG9504) has low identity with the homologue in *S. littoralis*, five amino acid residues, which are the potential contributors to substrate binding, are well conserved.

EO activity has been demonstrated in the midgut, fat body, haemolymph and integument of larvae in various insect species, although the predominant activity appears to be in midgut during the larval stage [10]. Our quantitative PCR results show that the mRNA for DmEO was highly expressed in the midgut during the last larval instar, and was expressed at a very low level in other tissues. These data indicate that the midgut is the main tissue that can produce EO during the larval stage, which corresponded with the situation in *S. littoralis*.

The developmental profile of the enzymic activity (Figure 6A) revealed that EO is predominantly expressed during the late stage of each larval instar. Similarly, quantitative PCR analysis revealed that the expression pattern of mRNA corresponds closely to the enzyme activity profile (Figure 6B). This result suggests that DmEO is primarily regulated at the transcriptional level. The fact that high expression of EO occurs in the late stage of each larval instar, when the active moulting hormone titre is high, suggests that the enzyme may play a role in modification of endogenous ecdysteroids. In *S. littoralis*, EO activity also increases at a similar developmental stage as observed in *D. melanogaster*.

In lepidopteran species, it has been shown that EO activity can be induced by the ecdysteroid agonists, RH-5849 or RH-5992, but not by 20E [10,42], possibly because exogenous 20E is rapidly inactivated. Similarly, in the current work, mRNA for DmEO is induced by the ecdysteroid agonist, RH-0345. Furthermore, *in vivo* expression of this enzyme seems to be stimulated by the endogenous ecdysteroids. Li and White [43] showed that the CG9504 expression during metamorphosis was significantly suppressed in the EcR (ecdysone receptor) mutant fly. Therefore DmEO is likely to be regulated by ecdysteroids. Also, these results indicate that the regulation mechanisms of EO are highly conserved between Diptera and Lepidoptera, in spite of the fact that the primary structure of the amino acid sequences shows diversity. This may mean that the precise control of EO expression is primarily important to achieve normal development in many insect species.

The question of the nature of the true active ecdysteroids has often been addressed [42,44], but not solved. Whereas ecdysone, 20E and their 3-dehydro derivatives exhibited different sensitivities in the *Drosophila* salivary gland chromosome-puffing assay, no stage-specific or locus-specific differential response to the four compounds was detected [45]. In contrast, evidence for possible specific roles of ecdysone and 20E has been obtained in other biological systems [46–48]. Furthermore, peripheral (target site) metabolism of ecdysteroids, taken in conjunction with the occurrence of multiple ecdysteroid receptors in a single insect species that might have different steroid-binding specificities, could conceivably result in differential effects of ecdysteroids. This would be analogous to control of vertebrate steroid hormone receptor loading by target-site steroid metabolism. Such control is exemplified by the reversible 3α-hydroxysteroid dehydrogenases, which work as molecular switches and control steroid hormone action [49]. This may mean that each endogenous ecdysteroid can have a distinct biological function in insects. In fact, it has been shown that 3D20E (3-dehydro-20-hydroxyecdysone) is much more efficient than 20E in induction of transcription of the steroid inducible *fbp1* gene in *Drosophila* [50] Additionally, 3D20E is a major endogenous metabolite in final-instar *Drosophila* larvae [51].

Recently, Baker et al. [52] proposed a novel, atypical ecdysteroid signalling pathway mediated by the orphan nuclear receptor, DHR38, in *Drosophila* that differs appreciably from the classical pathway mediated by binding of ecdysteroid to the EcR heterodimer with Ultraspiracle. In the DHR38 pathway, DHR38 heterodimerizes with Ultraspiracle, which must be pre-activated, but direct binding of ecdysteroids to either DHR38 or Ultraspiracle is not involved. Significantly, certain 3-dehydro-ecdysteroids and 3-epi-20-hydroxyecdysone showed high activity in the DHR38 pathway [52]. These data, taken together, may suggest that DmEO, which can use both ecdysone and 20E as substrates and produce 3-dehydroecdysteroids, plays a role not only in steroid ligand inactivation, but also steroid activation. Although we could not detect 3DE 3β-reductase activity in *D. melanogaster* under our conditions (H. Takeuchi, P. C. Turner and H. H. Rees, unpublished work), in several species and tissues, co-occurrence of EO and 3DE 3β-reductase activities constitutes an apparent 'futile cycle' (Figure 1) [2]. However, the demonstration of hormonal activity of 3-dehydro- and 3-epi-ecdysteroids in the DHR 38 pathway may provide an added significance to these steroid-transforming enzymic activities and to such a 'cycle'.

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