Regulation of ecdysteroid signalling: molecular cloning, characterization and expression of 3-dehydroecdysone 3α-reductase, a novel eukaryotic member of the short-chain dehydrogenases/reductases superfamily from the cotton leafworm, *Spodoptera littoralis*

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One route of inactivation of ecdysteroids in insects involves ecdysone oxidase-catalysed conversion into 3-dehydroecdysone (3DE), followed by irreversible reduction by 3DE 3α-reductase to 3-epiecdysone. The 3DE 3α-reductase has been purified and subjected to limited amino acid sequencing. It occurs as two distinct forms, including a probable trimer of subunit molecular mass of approx. 26 kDa. A reverse-transcriptase PCR-based approach has been used to clone the cDNA (1.2 kb) encoding the 26 kDa protein. Northern blotting showed that the mRNA transcript was expressed in Malpighian tubules during the early stage of the last larval instar. Conceptual translation of the 3DE 3α-reductase cDNA and database searching revealed that the enzyme belongs to the short-chain dehydrogenases/reductases superfamily. Furthermore, the enzyme is a novel eukaryotic 3-dehydrosteroid 3α-reductase member of that family, whereas vertebrate 3-dehydrosteroid 3α-reductases belong to the aldo-keto reductase (AKR) superfamily. Enzymically active recombinant 3DE 3α-reductase has been produced using a baculovirus expression system. Surprisingly, we observed no similarity between this 3DE 3α-reductase and a previously reported 3DE 3β-reductase, which acts on the same substrate and belongs to the AKR family.

Key words: insect hormone, moulting hormone, steroid reductase.

INTRODUCTION

Moulting and aspects of reproduction in insects are regulated by the steroidal moulting hormones (ecdysteroids) [1]. In the immature stages of insects, the prothoracic glands are the primary source of ecdysteroids, generally ecdysone (Scheme 1; **I**). However, in most Lepidoptera investigated, the major product of the glands is 3-dehydroecdysone (3DE; Scheme 1, **II**), accompanied by varying proportions of ecdysone [2–6]. The 3DE then undergoes reduction to ecdysone by NAD(P)H-linked 3DE 3β-reductase in the haemolymph [3,4,6,7]. Ecdysone undergoes 20-hydroxylation in certain peripheral tissues, yielding 20-hydroxyecdysone, which is considered to be the major active moulting hormone in most insect species [8].

The ecdysteroid titre exhibits obligatory, distinct peaks at specific stages in development [9]. In immature stages, these arise by increased ecdysteroid synthesis in the prothoracic glands, whereas decreases in titre result from enhanced ecdysteroid inactivation reactions together with elevated excretion. A number of transformations contribute to the inactivation of ecdysteroids [8], including the formation of 3-epi(3α-hydroxy)ecdysteroids, which are regarded as hormonally inactive [10–12]. Although production of 3-epiecdysteroids occurs in many insect orders, it is apparently prominent in lepidopteran midgut cytosol and involves ecdysone oxidase-catalysed formation of 3-dehydroecdysteroid, followed by NAD(P)H-dependent irreversible reduction to 3-epiecdysteroid (Scheme 1; **III**) [8,11–15]. The 3-dehydroecdysteroid may also undergo NAD(P)H-dependent
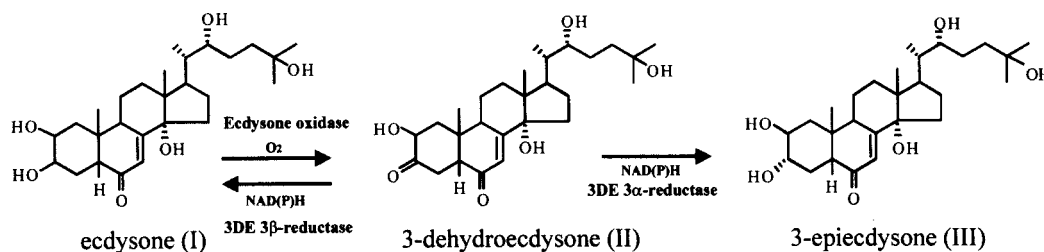
Scheme 1   Enzymic interconversions of ecdysone, 3-dehydroecdysone and 3-epiecdysone

3DE 3β-reductase-catalysed reduction back to 3β-hydroxysteroid (Scheme 1; reviewed in [8,16]). Similar reactions occur with ecdysone and 20-hydroxyecdysone. The significance of reduction of 3DE back to ecdysone is uncertain. In fact, particularly puzzling is the occurrence of enzymes for reversible inter-conversion of ecdysteroids and their 3-dehydro-derivatives in tissues of several species that are incapable of producing 3-epiecdysteroids [16].

We recently reported the purification of 3DE 3β-reductase from haemolymph of the cotton leafworm, *Spodoptera littoralis* [6]. Based upon limited amino acid sequences in this enzyme protein, its cDNA was cloned by a PCR-based approach. Conceptual translation and amino acid sequence analysis suggested that 3DE 3β-reductase from *S. littoralis* is a new member of the aldo-keto reductase (AKR) superfamily [17]. Furthermore, Northern-blot analysis showed that 3DE 3β-reductase mRNA transcripts are widely distributed in various tissues of *S. littoralis*.

As part of our studies aimed at elucidating the regulation of ecdysteroid titre and the reactions involved in ecdysteroid inactivation, we have purified 3DE 3α-reductase from midgut with attached Malpighian tubules of last instar larvae of *S. littoralis* [18]. Two forms of 3α-reductase were detected: a 26 kDa form, which may exist as a trimer with apparent molecular mass of 76 kDa, and a monomer with approximate molecular mass of 51 kDa. Limited amino acid sequences of internal proteolytic peptides of the 26 kDa form were determined, since the N-terminus is apparently blocked.

Here we report the molecular cloning and characterization of the cDNA encoding the approx. 26 kDa 3DE 3α-reductase of the cotton leafworm, *S. littoralis*, together with heterologous expression of enzymically active recombinant enzyme. Conceptual translation and amino acid sequence analysis indicated that 3DE 3α-reductase is closely related to members of the short-chain dehydrogenases/reductases (SDR) superfamily. In fact, the 3DE 3α-reductase is novel in being, hitherto, the only eukaryotic 3-dehydrosteroid 3α-reductase member of the SDR superfamily.

EXPERIMENTAL

Protein sequencing

3DE 3α-reductase (26 kDa form) from *S. littoralis* was purified as described previously [18]. To obtain limited amino acid sequences of the enzyme, purified protein was resolved by SDS/PAGE on 10 % gels, visualized by Coomassie staining, and the corresponding band was excised and cleaved with V8 proteinase. The resulting proteolytic peptides were purified by HPLC and sequenced by Edman degradation using an automated pulsed liquid-phase sequencer (Applied Biosystems 471A; Applied Biosystems, Foster City, CA, U.S.A.). The partial sequences of three proteolytic peptides were determined: fragment 1, SVAFVGRHQAXL (where X represents a gap); fragment 2, IAXMIVYLA; fragment 3, LAPSGVRVNXVNPGPVLTDI-AAG.

cDNA cloning and sequencing

A PCR-based cloning strategy was used to clone a cDNA fragment encoding the region between internal peptides sequenced as described above. Three degenerate primers were synthesized. Primer 3A26-1 was designed on the basis of a part of the internal proteolytic peptide fragment 1 amino acid sequence (5′-GCI TTY GTI GGI MGI CAY CAR GC, where I represents inosine, Y is T/C, M is A/C, and R is A/G); primer 3A26-3 and 3A26-3AS were designed according to the sequence of internal proteolytic peptide fragment 3 (3A26-3, 5′-GCI CCI WSI GGI GTI MGI GTN AA, where W represents A/T, S is C/G, N is
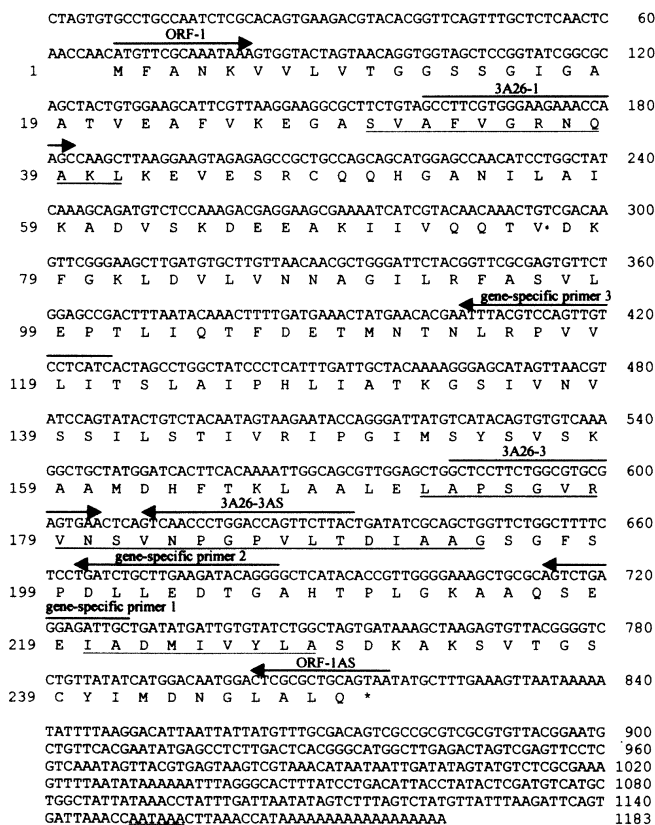
A/T/C/G; reverse primer 3A26-3AS, 5′-GTI ARI ACI GGI CCI GGR TTN AC).

Total RNA was extracted using TRIzol® (Life Technologies) from combined midgut and Malpighian tubules dissected from larvae 42 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, Lewes, East Sussex, U.K.) with Q_T adapter primer, 5′-CCA TCA GTG CTA GAC AGC TAA GCT TGA GCT CGG ATC C(T)$_{17}$ (modified from [19]). cDNA synthesized with Q_T primer served as template for the PCR, in which the above three degenerate primers were combined in turn with the adapter Q_0 primer, 5′-CCA TCA GTG CTA GAC AGC T (modified from [19]). PCR was carried out as follows: 1 cycle of 94 °C for 3 min and 35 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 2 min and 1 cycle of 72 °C for 8 min. PCR products were analysed by electrophoresis on a 1 % agarose gel. This revealed that PCR with 3A26-1 and Q_0 yielded various lengths of product. These PCR products were 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 2 min and 1 cycle of 72 °C for 8 min. The nested PCR with 3A26-3 and the other adapter $Q_{1\cdot2}$ primer, 5′-TAA GCT TGA GCT CGG A (modified from [19]), yielded a product of approx. 550 bp, and the semi-nested PCR with 3A26-1 and 3A26-3AS yielded a product of approx. 450 bp. Both PCR products were gel-purified using a Hybaid Recovery™ DNA purification kit (Hybaid, Teddington, Middx., U.K.).

The purified PCR products were cloned into pGEM®-T Easy Vector (Promega). Transformants were screened by colony-PCR using M13 forward primer and M13 reverse primer (5′-GTA AAA CGA CGG CCA G and 5′-CAG GAA ACA GCT ATG AC respectively). Those showing the correct size of insert were propagated in Luria–Bertani 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). The sequences of three independent clones of both cloned products 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 the Dynabeads® mRNA purification kit (Dynal, Bromborough, Merseyside, U.K.). A 5′-RACE System Version 2.0 (Life Technologies) was used to amplify the 5′-terminus of the message for sequencing. Briefly, a gene-specific primer 1 (5′-GCA ATC TCC TCA GAC T) 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′-CCC TGT ATC TTC AAG CAG ATC A and 5′-GAT GAG GAC AAC TGG ACG TAA AT respectively), together with the anchor primers supplied by the manufacturer. The second-round PCR yielded a product of approx. 400 bp, which was cloned into pGEM®-T Easy Vector, and the nucleotide sequences of several clones were determined.

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CTAGTGTGCCTGCCAATCTCGCACAGTGAAGACGTACACGGTTCAGTTTGCTCTCAACTC      60
          ORF-1
    AACCAACATGTTCGCAAATAAAGTGGTACTAGTAACAGGTGGTAGCTCCGGTATCGGCGC    120
1         M  F  A  N  K  V  V  L  V  T  G  G  S  S  G  I  G  A
                                                         3A26-1
    AGCTACTGTGGAAGCATTCGTTAAGGAAGGCGCTTCTGTGGCCTTCGTGGGAAGAAACCA    180
19  A  T  V  E  A  F  V  K  E  G  A  S  V  A  F  V  G  R  N  Q
    AGCCAAGCTTAAGGAAGTAGAGAGCCGCTGCCAGCAGCATGGAGCCAACATCCTGGCTAT    240
39  A  K  L  K  E  V  E  S  R  C  Q  Q  H  G  A  N  I  L  A  I
    CAAAGCAGATGTCTCCAAAGACGAGGAAGCGAAAATCATCGTACAACAAACTGTCGACAA    300
59  K  A  D  V  S  K  D  E  E  A  K  I  I  V  Q  Q  T  V· D  K
    GTTCGGGAAGCTTGATGTGCTTGTTAACAACGCTGGGATTCTACGGTTCGCGAGTGTTCT    360
79  F  G  K  L  D  V  L  V  N  N  A  G  I  L  R  F  A  S  V  L
                                          gene-specific primer 3
    GGGAGCCGACTTTAATACAAACTTTTGATGAAACTATGAACACGAATTTACGTCCAGTTGT    420
99  E  P  T  L  I  Q  T  F  D  E  T  M  N  T  N  L  R  P  V  V
    CCTCATCACTAGCCTGGCTATCCCTCATTTGATTGCTACAAAAGGGAGCATAGTTAACGT    480
119 L  I  T  S  L  A  I  P  H  L  I  A  T  K  G  S  I  V  N  V
    ATCCAGTATACTGTCTACAATAGTAAGAATACCAGGGATTATGTCATACAGTGTGTCAAA    540
139 S  S  I  L  S  T  I  V  R  I  P  G  I  M  S  Y  S  V  S  K
    GGCTGCTATGGATCACTTCACAAAATTGGCAGCGTTGGAGCTGGCTCCTTCTGGCGTGCG    600
159 A  A  M  D  H  F  T  K  L  A  A  L  E  L  A  P  S  G  V  R
                    3A26-3AS
    AGTGAACTCAGTCAACCCTGGACCAGTTCTTACTGATATCGCAGCTGGTTCTGGCTTTTC    660
179 V  N  S  V  N  P  G  P  V  L  T  D  I  A  A  G  S  G  F  S
            gene-specific primer 2
    TCCTGATCTGCTTGAAGATACAGGGGCTCATACACCGTTGGGGAAAGCTGCGCAGTCTGA    720
199 P  D  L  L  E  D  T  G  A  H  T  P  L  G  K  A  A  Q  S  E
    gene-specific primer 1
    GGAGATTGCTGATATGATTGTGTATCTGGCTAGTGATAAAGCTAAGAGTGTTACGGGGTC    780
219 E  I  A  D  M  I  V  Y  L  A  S  D  K  A  K  S  V  T  G  S
                              ORF-1AS
    CTGTTATATCATGGACAATGGACTCGCGCTGCAGTAATATGCTTTGAAAGTTAATAAAAA    840
239 C  Y  I  M  D  N  G  L  A  L  Q  *
    TATTTTAAGGACATTAATTATTATGTTTGCGACAGTCGCCGCGTCGCGTGTTACGGAATG    900
    CTGTTCACGAATATGAGCCTCTTGACTCACGGGCATGGCTTGAGACTAGTCGAGTTCCTC     960
    GTCAAATAGTTACGTGAGTAAGTCGTAAACATAATAATTGATATAGTATGTCTCGCGAAA   1020
    GTTTTAATATAAAAAATTTAGGGCACTTTATCCTGACATTACCTATACTCGATGTCATGC   1080
    TGGCTATTATAAACCTATTTGATTAATATAGTCTTTAGTCTATGTTATTTAAGATTCAGT   1140
    GATTAAACCAATAAACTTAAACCATAAAAAAAAAAAAAAAAAA                    1183
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Figure 1 Nucleotide and deduced amino acid sequences of 3DE 3α-reductase

Nucleotide numbers are indicated on the right, amino acid numbers are on the left. The putative polyadenylation signal is double-underlined. The amino acid sequences obtained from three V8 proteolytic peptides derived from purified 3DE 3α-reductase are underlined. The termination codon is indicated by an asterisk. The positions and directions of primers are indicated as arrows above the nucleotide sequence.

Construction of a cDNA containing an open reading frame

The cDNA sequence containing the entire open reading frame was amplified by PCR from the cDNA synthesized with primer Q_T as described above. Expand™ High Fidelity PCR System (Roche Molecular Biochemicals) was used with the following gene-specific primers: ORF-1 (5′-TCA GAATTCATG TTC GCA AAT AAA) and ORF-1AS (5′-CAA AGATCTTTA CTG CAG CGC GAG). These primers were designed to incorporate EcoRI, and BglII sites (underlined in the primer sequences) into the 5′-end and 3′-end of the sense strand respectively. PCR was conducted as follows: 1 cycle of 94 °C for 3 min, 37 cycles of 94 °C for 1 min, 51 °C for 1 min, 68 °C for 1 min and 1 cycle of 72 °C for 10 min. The resulting PCR product was gel-purified, and cloned into pGEM®-T Easy Vector. Three independent clones were sequenced.

Northern-blot analysis

Total RNA from various tissues was isolated using TRIzol® reagent. A 10 μg aliquot of RNA was fractionated on a formaldehyde/agarose gel, transferred to Electran® nylon membrane (Merck), and hybridized with a probe corresponding to the whole open reading frame of the 3DE 3α-reductase cDNA. The probe was radiolabelled with ^{32}P using a Ready-To-Go™ DNA labelling kit (-dCTP) (Amersham Pharmacia Biotech), and

loading was normalized by probing with an 18 S rRNA probe from mouse (a gift from Ms Yi-Ping Bao, University of Liverpool, U.K.). Prehybridization and hybridization were carried out using QuickHyb® hybridization solution (Stratagene), under the conditions recommended by the manufacturer. The blots were washed at high stringency at 60 °C with $0.1 \times$ SSC (where $1 \times$ SSC is 0.15 M NaCl/0.015 M sodium citrate) containing 0.1 % SDS, and labelled bands were visualized by autoradiography.

Baculovirus expression of 3DE 3α-reductase

The cDNA containing the complete open reading frame of 3DE 3α-reductase was cut out with EcoRI and BglII from the plasmid as described above and transferred into pSynXIV VI+X3 [20], which was digested with the same enzymes. This was cotransfected into S. frugiperda Sf21 cells (approx. 2×10^6 cells) with vSynVI−gal DNA [20]. Recombination between viral sequences flanking the 3DE 3α-reductase 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 3DE 3α-reductase 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 and Southern blotting. Procedures used for maintenance of Sf21 cells, for propagation of vSynVI−gal, and for the construction and characterization of recombinant viruses are as described in [20].

SDS/PAGE

Total cell proteins were analysed by vertical 4–12 % gradient acrylamide gel electrophoresis using the NuPAGE Bis-Tris system (Novex, Groningen, The Netherlands). All samples (from approx. 3×10^5 cells each) were lysed in 50 μl of the NuPAGE SDS sample buffer containing NuPAGE sample reducing agent, and boiled for 5 min before loading 5 μl of each sample onto the gel. After electrophoresis, the gel was stained with Coomassie Brilliant Blue and destained to allow visualization of the proteins. The band of recombinant protein was cut out and subjected to in-gel digestion with trypsin and HPLC purification, before limited sequencing.

Enzyme assay

Assay of the recombinant 3DE 3α-reductase activity was performed in duplicate by modification of the method described in [14]. Sf21 cells (3×10^5 cells/well), infected with wild-type Autographa californica nuclear polyhedrosis strain baculovirus or with recombinant baculovirus, were homogenized at 68 h post-infection in 50 μl of 100 mM Tris buffer (pH 8.0) containing 0.05 % NaN_3. A 20 μl aliquot of this extract was assayed by incubation for 30 min at 37 °C in a 50 μl assay mixture consisting of 0.1 M sodium phosphate buffer (pH 7.4), 0.5 mM NADPH and 60 μM 3DE. Assays were quenched with 50 μl of methanol, and proteins were removed by centrifugation. The supernatant was analysed by reversed-phase HPLC. Protein concentration was determined by the method of Bradford using a dye-binding assay (Bio-Rad), with BSA as a standard. Three independent experiments were performed for each assay, with all assays being carried out in duplicate.

HPLC

Ecdysteroids were analysed by HPLC on a Waters instrument (Waters Associates, Northwich, Cheshire, U.K.) linked to a 440

UV detector set at 254 nm and a C_{18} Nova-Pak cartridge (10 cm × 5 mm; Waters Associates), 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 [14].

RESULTS

Cloning of the cDNA encoding 3DE 3α-reductase

A PCR-based cloning strategy, as detailed in the Experimental section, allowed us to obtain two cDNA fragments corresponding to the sequences between nt 161–633 and 584–1183 respectively (Figure 1). Gene-specific primers derived from these sequences were synthesized and used for 5′-RACE to obtain the 5′-end of the cDNA. 5′-RACE produced a cDNA clone of 427 bp which contains a putative translation start site at position 68. Taken together, all overlapping cDNAs span a total of 1183 bp. The polyadenylation signal (AATAAA) is located at position 1150. As shown in Figure 1, using the first ATG as the start codon, the full-length 3DE 3α-reductase cDNA encodes a protein of 249

amino acids with a predicted molecular mass of 26079.09, which is almost identical to its apparent M_r observed in our SDS/PAGE analysis [18]. The protein is mildly acidic with an estimated pI of 5.82.

Similarity of the deduced amino acid sequence to proteins of the SDR family

The deduced amino acid sequence for the cDNA coding region showed similarity to enzymes in the SDR family (Figure 2), such as *Drosophila melanogaster* alcohol dehydrogenase (69 %), *Bacillus megaterium* glucose 1-dehydrogenase II (66 %), *Cuphea lanceolata* 3-oxoacyl-reductase (61 %) and *Comamonas testosteroni* 3β-hydroxysteroid dehydrogenase (58 %). The 3DE 3α-reductase amino acid sequence exhibited features characteristic of the proteins of the SDR family [21,22], such as the putative consensus sequence for the cofactor binding site [23,24], $G(X)_3GXG$ at Gly^{11}, the active site consensus sequence [25–27], $Y(X)_3K$ at Tyr^{154}, and three further residues (Gly^{133}, Ser^{140} and
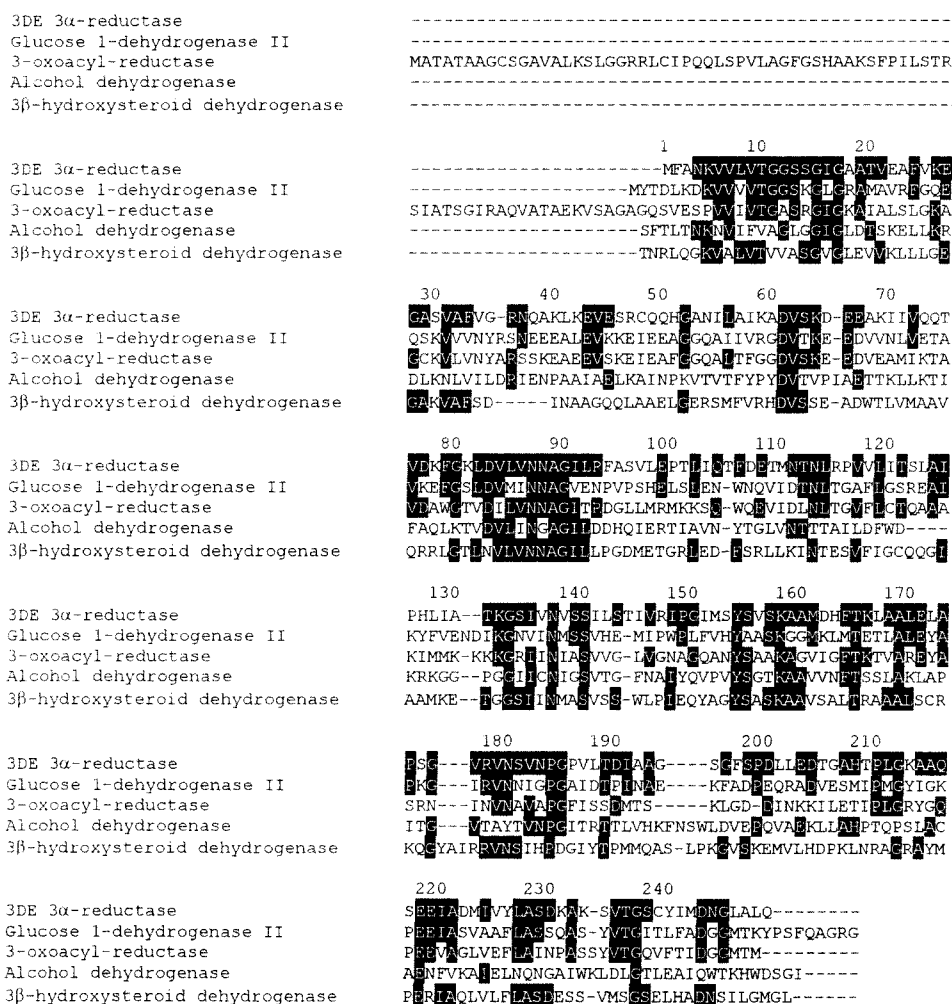

Figure 2   Alignment of the deduced amino acid sequences of 3DE 3α-reductase and the most similar database proteins

The deduced 3DE 3α-reductase sequence was compared with all sequences in the Swiss-Prot database using the BLAST programme. Only the amino acid sequences of some similar proteins representing the SDR family are shown in the alignment. Gaps introduced to optimize alignment are indicated by hyphens. Identical amino acids between 3DE 3α-reductase and at least one other sequence are indicated in black boxes. Numbers on the top refer to the amino acid residues of the 3DE 3α-reductase sequence. The accession number of each protein sequence is as follows: *B. megaterium* glucose 1-dehydrogenase II, P39483; *C. lanceolata* 3-oxoacyl-reductase, P28643; *D. melanogaster* alcohol dehydrogenase, P00334; and *C. testosteroni* 3β-hydroxysteroid dehydrogenase, P19871. The alignment was constructed by use of the CLUSTALW programme.
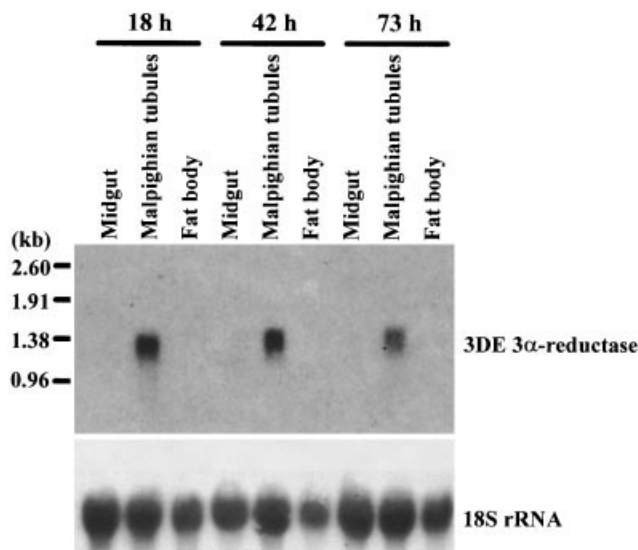
Figure 3   Northern-blot analysis of the tissue distribution and expression of 3DE 3α-reductase

A 10 μg aliquot of total RNA from various tissues at different times (top) within the last larval instar was used. The blot was hybridized with a ^{32}P-labelled 3DE 3α-reductase cDNA probe which corresponded to the whole protein coding region. The same blot was stripped and reprobed with 18 S rRNA probe. The positions of RNA size markers are shown on the left.
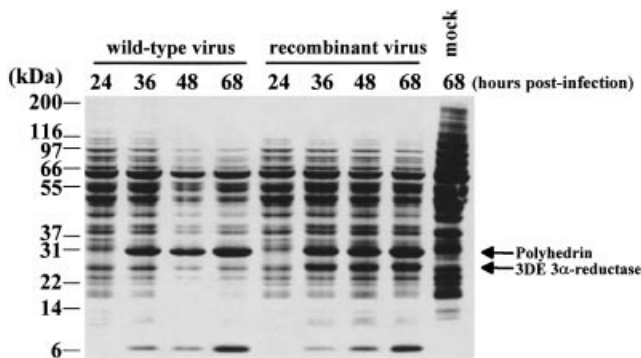

Figure 4   SDS/PAGE analysis of protein synthesis in Sf21 cells infected with recombinant and wild-type baculovirus

Infected cells (approx. 3×10^5 cells per sample) were lysed and collected at 24, 36, 48 and 68 h post-infection. Each extract (10% of the total) was analysed by SDS/PAGE and visualized by Coomassie Brilliant Blue (see the Experimental section). The positions of protein molecular weight markers are shown on the left. The positions of 3DE 3α-reductase and polyhedrin are shown on the right.

Pro[184]) which are highly conserved in members of that super-family [21].

Tissue distribution and developmental expression of 3DE 3α-reductase mRNA

As demonstrated in Figure 3, a cDNA probe representing the protein coding region detected a transcript of approx. 1.2 kb in the Malpighian tubules prepared from larvae at 18, 42 and 73 h into the last-larval instar. No detectable expression was found in fat body and midgut. We could not detect the mRNA of 3α-reductase in haemocytes at 42 h (results not shown).
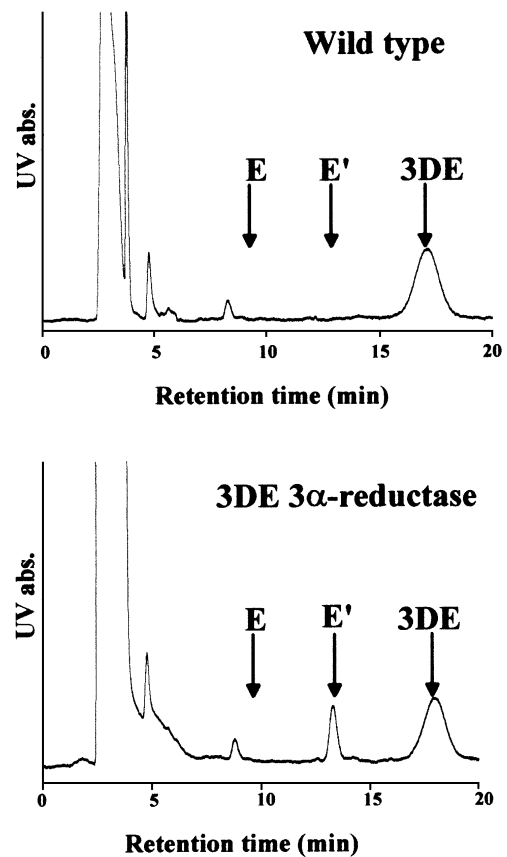
Figure 5   Enzymic activity of recombinant 3DE 3α-reductase

Sf21 cells infected with wild-type or recombinant virus were lysed, incubated with 3-dehydroecdysone in the presence of NADPH 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 3-dehydroecdysone (3DE) are shown.

Expression of 3DE 3α-reductase in Sf21 cells

Monolayers of Sf21 cells (3×10^5 cells/well) were infected with wild-type or with recombinant virus, and the cells were collected at intervals between 24 and 68 h. It was found that a polypeptide of approx. 26 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 (Figure 4). This recombinant protein was con-firmed to be the 3α-reductase by in-gel digestion with trypsin followed by sequencing of two internal fragments (VVLVT-GGXXGIG and KXXALXLAP). These correspond to amino acids 6–17 and 166–174 in the 3DE 3α-reductase sequence. The level of expression of 3DE 3α-reductase is comparable with that of viral polyhedrin protein at approx. 30 kDa.

It was demonstrated that the recombinant protein cell lysate was enzymatically functional in reduction of 3-dehydroecdysone to 3-epiecdysone in the presence of NADPH as a cofactor (Figure 5). As shown in the reversed-phase HPLC UV chromatograms (Figure 5), there was appreciable conversion of 3-dehydro-ecdysone into 3-epiecdysone by the NADPH-supplemented lysate from 3DE 3α-reductase recombinant virus-infected cells, with no detectable transformation in the case of wild-type virus-infected cells (Figure 5) or lysate of uninfected Sf21 cells alone (results not shown). In the case of the 3DE 3α-reductase recombinant virus-infected cell lysate, the 3DE 3α-reductase activity was absolutely

dependent upon NAD(P)H cofactor and the specific activity was 2.86 ± 0.32 nmol/min per mg of protein in the presence of NADPH (mean $\pm$ S.E.M. for three independent experiments), which is of the same order of magnitude as partially fractionated normal activity [18].

DISCUSSION

Using a reverse-transcription PCR-based cloning strategy, employing degenerate primers designed on the basis of the partial amino acid sequences of fragments of 3DE 3α-reductase, together with 5′-RACE, the complete cDNA encoding the enzyme was isolated and sequenced. The predicted amino acid sequence of the cDNA contained the three internal peptides obtained from V8 proteinase digestion of the band corresponding to the M_r 26000 polypeptide, confirming that we had cloned the 3DE 3α-reductase cDNA.

When the cDNA encoding 3DE 3α-reductase was expressed using the baculovirus system, a polypeptide band of approx. 26 kDa was observed on SDS/PAGE which increased in intensity with time of culture (Figure 4). Limited sequencing of internal proteolytic fragments confirmed that the sequences corresponded to the conceptually translated amino acid sequence of the cDNA. Furthermore, the lysate from recombinant baculovirus-infected cells showed NADPH-dependent 3DE 3α-reductase enzymic activity. The foregoing results indicate that the cloned cDNA encodes 3DE 3α-reductase.

Database searching revealed that 3DE 3α-reductase belongs to the SDR superfamily [21,22]. It is clear that the predicted amino acid sequence of this enzyme has high similarity to other enzymes in this family; in particular, the glycine residues (Gly[11], Gly[15] and Gly[17]) involved in cofactor binding [23,24], as well as Tyr[154] and Lys[158] essential for catalytic function, are conserved [25–27]. Furthermore, that 3DE 3α-reductase may exist as a homotrimer of apparent molecular mass approx. 76 kDa and subunit molecular mass approx. 26 kDa [18] is in agreement with SDR proteins, which are homo-oligomers composed of subunits with molecular masses between 25 kDa and 37 kDa [22].

3DE 3α-reductase activity has been demonstrated in midgut, fat body, haemolymph and epidermis of various insect species [11,14,28,29], but hitherto, there were no data concerning this enzyme in Malpighian tubules. Our Northern-blot analysis has revealed that the mRNA for the 3α-reductase is expressed strongly in Malpighian tubules (Figure 3). Furthermore, we have detected enzymic activity of the 3α-reductase in Malpighian tubules (results not shown). Chen et al. [18] showed that there were at least two forms (26 kDa and 51 kDa) of the 3α-reductase in combined midgut and Malpighian tubules of *S. littoralis*. The current data suggest that expression of the small form of 3α-reductase occurs in the Malpighian tubules, but not in the midgut. Expression of the 3α-reductase in the Malpighian tubules may suggest a role for the enzyme in inactivation of endogenous haemolymph ecdysteroid.

Northern-blot analysis (Figure 3) also revealed that the mRNA transcript for the enzyme is expressed over 18–73 h of the last larval instar. Since this expression correlates with times in development when ecdysteroid titres are minimal [14], the 3α-reductase may function in keeping the hormone levels low. In final larval instar of the Lepidoptera, *Manduca sexta* [30], *Diatraea grandiosella* [31] and *Bombyx mori* [32], very low multiple peaks in haemolymph ecdysteroid titre have been detected appreciably earlier in development than the commitment peak. Presumably, a similar situation exists in *S. littoralis*. Thus expression of the mRNA encoding the small form of 3α-reductase in the early stage of the last larval instar may reflect involvement

of the enzyme in inactivation of such small ecdysteroid peaks in the Malpighian tubules.

Hydroxysteroid dehydrogenases (HSDs) belong to at least two distinct protein families: the SDR and AKR families [21,22,33]. Furthermore, some 3β-HSDs belong to an additional 3β-HSD family whose members are membrane-bound proteins located in the endoplasmic reticulum and mitochondrial membranes [34]. HSDs belonging to the SDR or AKR families interconvert similar substrates, but bear no significant sequence similarity, and have completely different three-dimensional structures [35,36].

Recently, we reported [17] the deduced amino acid sequence of another reductase enzyme from *S. littoralis* that reduces 3DE to yield a 3β-hydroxy-ecdysteroid (biologically active), rather than a 3α-hydroxy-ecdysteroid (inactive), as in the case of the 3DE 3α-reductase. Surprisingly, the 3DE 3β-reductase belongs to the AKR superfamily [33], whereas the current 3DE 3α-reductase belongs to the SDR superfamily [21,22]. Furthermore, there are no conserved regions between these families, resulting in no significant sequence similarity. Within the SDR superfamily, members share a consensus active site sequence Tyr-X-X-X-Lys, the tyrosine and lysine residues being essential for catalysis [21]. Thus it is envisaged that Tyr[154] and Lys[158] are important residues at the active site of 3DE 3α-reductase, whereas Asp[73], Tyr[78], Lys[107], and His[140] in the 3DE 3β-reductase [17] are characteristic of active sites of the AKR superfamily [35]. SDR superfamily members have a Rossmann nucleotide-binding fold [G(X)$_3$GXG] located near the N-terminus [21,24]. In contrast, AKR family members bind cofactors through interaction with residues at the C-terminus end, consisting of an α/β barrel or several β-strands and α-helix [36]. Thus, based on conserved amino acid residues in these superfamilies, we expect the structure of 3DE 3α-reductase to be very different from that of the corresponding 3DE 3β-reductase, though they catalyse reduction of an identical substrate. Furthermore, we found no nucleotide sequence similarity between the cDNA for these two reductases, which supports our view that they have evolved independently.

Surprisingly, our protein database and literature searches have revealed that no other 3-dehydrosteroid 3α-reductase enzyme hitherto reported belongs to the SDR family, except for bacterial ones [37,38]. Mammalian 3α-HSDs belong to the AKR superfamily [22,39–42], whereas carbonyl reductases from mammalian tissues belong to the SDR family [43–46]. These results may suggest that 3DE 3α-reductase from *S. littoralis* is not evolutionarily close to 3α-HSDs but to carbonyl reductases in mammals. It may be significant in the case of ecdysteroids that a specific ecdysone oxidase (i.e. oxygen-dependent), rather than an NAD(P)$^+$-dependent dehydrogenase, is responsible for oxidation of ecdysteroid to 3-dehydroecdysone, whereas the vertebrate 3-HSDs generally catalyse reversible reactions.

REFERENCES

1   Koolman, J. (1989) Ecdysone, Georg Thieme-Verlag, Stuttgart, Germany
2   Warren, J. T., Sakurai, S., Rountree, D. B., Gilbert, L. I., Lee, S. S. and Nakanishi, K. (1988) Regulation of the ecdysteroid titer of *Manduca sexta*: reappraisal of the role of the prothoracic glands. Proc. Natl. Acad. Sci. U.S.A. **85**, 958–962
3   Sakurai, S., Warren, J. and Gilbert, L. I. (1989) Mediation of ecdysone synthesis in *Manduca sexta* by a hemolymph enzyme. Arch. Insect Biochem. Physiol. **10**, 179–197
4   Kiriishi, S., Rountree, D. B., Sakurai, S. and Gilbert, L. I. (1990) Prothoracic gland synthesis of 3-dehydroecdysone and its hemolymph 3β-reductase mediated conversion to ecdysone in representative insects. Experientia **46**, 57–64

5 Blais, C. and Lafont, R. (1991) Biosynthesis of ecdysteroids by prothoracic glands in *Pieris brassicae* (Lepidoptera insect). Conversion *in vitro* of radiolabelled precursor of 3-dehydroecdysone. Acad. Sci. Paris C. R. Ser. III **313**, 359–364

6 Chen, J.-H., Webb, T. J., Powls, R. and Rees, H. H. (1996) Purification and characterisation of haemolymph 3-dehydroecdysone 3β-reductase in relation to ecdysteroid biosynthesis in the cotton leafworm *Spodoptera littoralis*. Eur. J. Biochem. **242**, 394–401

7 Watson, R. D., Spaziani, E. and Bollenbacher, W. E. (1989) Regulation of ecdysteroid biosynthesis in insects and crustaceans: a comparison. In Ecdysone (Koolman, J., ed.), pp. 188–196, Georg Thieme-Verlag, Stuttgart, Germany

8 Rees, H. H. (1995) Ecdysteroid biosynthesis and inactivation in relation to function. Eur. J. Entomol. **92**, 9–39

9 Steel, C. G. H. and Vafopoulou, X. (1989) Ecdysteroid titer profiles during growth and development of arthropods. In Ecdysone (Koolman, J., ed.), pp. 221–231, Georg Thieme-Verlag, Stuttgart, Germany

10 Nigg, H. N., Svoboda, J. A., Thompson, M. J., Kaplanis, J. N., Dutky, S. R. and Robbins, W. E. (1974) Ecdysone metabolism: ecdysone dehydrogenase-isomerase. Lipids **9**, 971–974

11 Blais, C. and Lafont, R. (1984) Ecdysteroid metabolism by soluble enzymes from an insect. Metabolic relationships between 3β-hydroxy-, 3α-hydroxy- and 3-oxoecdysteroids. Hoppe Seyler's Z. Physiol. Chem. **365**, 809–817

12 Milner, N. P. and Rees, H. H. (1985) Involvement of 3-dehydroecdysone in the 3-epimerization of ecdysone. Biochem. J. **231**, 369–374

13 Weirich, G. F. (1989) Enzymes involved in ecdysone metabolism. In Ecdysone (Koolman, J., ed.), pp. 174–180, Georg Thieme-Verlag, Stuttgart, Germany

14 Webb, T. J., Powls, R. and Rees, H. H. (1995) Enzymes of ecdysteroid transformation and inactivation in the midgut of the cotton leafworm, *Spodoptera littoralis*: properties and developmental profiles. Biochem. J. **312**, 561–568

15 Webb, T. J., Powls, R. and Rees, H. H. (1996) Characterization, fractionation and kinetic properties of the enzymes of ecdysteroid 3-epimerization and phosphorylation isolated from the midgut cytosol of the cotton leafworm, *Spodoptera littoralis*. Insect Biochem. Mol. Biol. **26**, 809–816

16 Lafont, R. and Koolman, J. (1984) Ecdysone metabolism. In Biosynthesis, Metabolism and Mode of Action of Invertebrate Hormones (Hoffmann, J. and Porchet, M., eds.), pp. 196–226, Springer-Verlag, Berlin

17 Chen, J.-H., Turner, P. C. and Rees, H. H. (1999) Molecular cloning and characterization of hemolymph 3-dehydroecdysone 3β-reductase from the cotton leafworm, *Spodoptera littoralis*. A new member of the third superfamily of oxidoreductases. J. Biol. Chem. **274**, 10551–10556

18 Chen, J.-H., Powls, R., Rees, H. H. and Wilkinson, M. C. (1999) Purification of ecdysone oxidase and 3-dehydroecdysone 3α-reductase from the cotton leafworm, *Spodoptera littoralis*. Insect Biochem. Mol. Biol. **29**, 899–908

19 Frohman, M. A. (1993) Rapid amplification of complementary DNA ends for generation of full-length complementary DNAs: Thermal RACE. Methods Enzymol. (Wu, R., ed.), **218**, 340–356

20 O'Reilly, D. R., Miller, L. K. and Luckow, V. A. (1994) Baculovirus Expression Vectors, Oxford University Press, New York, Oxford

21 Jörnvall, H., Persson, B., Krook, M., Atrian, S., Gonzàlez-Duarte, R., Jeffery, J. and Ghosh, D. (1995) Short-chain dehydrogenases/reductases (SDR). Biochemistry **34**, 6003–6013

22 Penning, T. M., Bennett, M. J., Smith-Hoog, S., Schlegel, B. P., Jez, J. M. and Lewis, M. (1997) Structure and function of 3α-hydroxysteroid dehydrogenase. Steroids **62**, 101–111

23 Wierenga, R. K., Maeyer, M. C. H. and Hol, W. G. J. (1985) Interaction of pyrophosphate moieties with α-helixes in dinucleotide binding-proteins. Biochemistry **24**, 1346–1357

24 Rossmann, M. G., Liljas, A., Brändén, C.-I. and Banaszak, L. J. (1975) Evolutionary and structural relationships among dehydrogenases. In The Enzymes (Boyer, P. D., ed.), Vol. 11, pp. 61–102, Academic Press, New York

25 Ghosh, D., Weeks, C. M., Grochulski, P., Duax, W. L., Erman, M., Rimsay, R. L. and Orr, J. C. (1991) Three-dimensional structure of holo 3α,20β-hydroxysteroid dehydrogenase: a member of a short-chain dehydrogenase family. Proc. Natl. Acad. Sci. U.S.A. **88**, 10064–10068

26 Ghosh, D., Wawrzak, Z., Weeks, C. M., Duax, W. L. and Erman, M. (1994) The refined three-dimensional structure of 3α,20β-hydroxysteroid dehydrogenase and possible roles of the residues conserved in short-chain dehydrogenases. Structure **2**, 629–640

27 McKinley-McKee, J. S., Winberg, J.-O. and Pettersson, G. (1991) Mechanism of action of *Drosophila melanogaster* alcohol-dehydrogenase. Biochem. Int. **25**, 879–885

28 Weirich, G. F., Feldlaufer, M. F. and Svoboda, J. A. (1993) Ecdysone oxidase and 3-oxoecdysteroid reductases in *Manduca sexta*: Developmental changes and tissue distribution. Arch. Insect Biochem. Physiol. **23**, 199–211

29 Thompson, M. J., Weirich, G. F. and Svoboda, J. A. (1985) Ecdysone 3-epimerase. Methods Enzymol. **111**, 437–442

30 Wolfgang, W. J. and Riddiford, L. M. (1986) Larval cuticular morphogenesis in the tabacco hornworm, *Manduca sexta*, and its hormonal regulation. Dev. Biol. **113**, 305–316

31 Yin, C.-M. and Chaw, W.-D. (1984) Hemolymph ecdysteroid titer and epidermal cell commitment of the female southwestern corn borer larvae. J. Insect Physiol. **30**, 257–263

32 Sakurai, S., Kaya, M. and Satake, S. (1998) Hemolymph ecdysteroid titer and ecdysteroid-dependent developmental events in the last-larval stadium of the silkworm, *Bombyx mori*: role of low ecdysteroid titer in larval-pupal metamorphosis and a reappraisal of the head critical period. J. Insect Physiol. **44**, 867–881

33 Bohren, K. M., Bullock, B., Wermuth, B. and Gabbay, K. H. (1989) The aldo-keto reductase superfamily. cDNAs and deduced amino acid sequences of human aldehyde and aldose reductases. J. Biol. Chem. **264**, 9547–9551

34 Labrie, F., Simard, J., Luu-The, V., Pelletier, G., Belanger, A., Lanchance, Y., Zhao, H. F., Labrie, C., Breton, N., DE Launoit Y. et al. (1992) Structure and tissue-specific expression of 3β-hydroxysteroid dehydrogenase/5-ene-4-ene isomerase genes in human and rat classical and peripheral steroidogenic tissues. J. Steroid Biochem. **41**, 421–435

35 Hoog, S. S., Pawlowski, J. E., Alzari, P. M., Penning, T. M. and Lewis, M. (1994) Three-dimensional structure of rat liver 3α-hydroxysteroid/dihydrodiol dehydrogenase: a member of the aldo-keto reductase superfamily. Proc. Natl. Acad. Sci. U.S.A. **91**, 2517–2521

36 Bennett, M. J., Schlegel, B. P., Jez, J. M., Penning, T. M. and Lewis, M. (1996) Structure of 3α-hydroxysteroid/dihydrodiol dehydrogenase complexed with NADP+. Biochemistry **35**, 10702–10711

37 Suzuki, K., Ueda, S., Sugiyama, M. and Imamura, S. (1993) Cloning and expression of a *Pseudomonas* 3α-hydroxysteroid dehydrogenase-encoding gene in *Escherichia coli*. Gene **130**, 137–140

38 Oppermann, U. C. T. and Maser, E. (1996) Characterization of a 3α-hydroxysteroid dehydrogenase/carbonyl reductase from the gram-negative bacterium *Comamonas testosteroni*. Eur. J. Biochem. **241**, 744–749

39 Stolz, A., Rahimi-Kiani, M., Ameis, D., Chan, E., Ronk, M. and Shively, J. E. (1991) Molecular structure of rat hepatic 3α-hydroxysteroid dehydrogenase. A member of the oxidoreductase gene family. J. Biol. Chem. **266**, 15253–15257

40 Cheng, K. C., White, P. C. and Qin, K. N. (1991) Molecular cloning and expression of rat liver 3α-hydroxysteroid dehydrogenase. Mol. Endocrinol. **56**, 823–828

41 Pawlowski, J. E., Huizinga, M. and Penning, T. M. (1991) Cloning and sequencing of the cDNA for rat liver 3α-hydroxysteroid/dihydrodiol dehydrogenase. J. Biol. Chem. **266**, 8820–8825

42 Penning, T. M. (1997) Molecular endocrinology of hydroxysteroid dehydrogenases. Endocrine Rev. **18**, 281–305

43 Wermuth, B., Bohren, K. M., Heinemann, G., Von Wartburg, J.-P. and Gabbay, K. H. (1988) Human carbonyl reductase. Nucleotide sequence analysis of a cDNA and amino acid sequence of the encoded protein. J. Biol. Chem. **263**, 16185–16188

44 Wei, J., Dlouhy, S. R., Hara, A., Ghetti, B. and Hodes, M. E. (1996) Cloning a cDNA for carbonyl reductase (Cbr) from mouse cerebellum: murine genes that express cbr map to chromosomes 16 and 11. Genomics **34**, 147–148

45 Nakanishi, M., Deyashiki, Y., Nakayama, T., Sato, K. and Hara, A. (1993) Cloning and sequence analysis of a cDNA encoding tetrameric carbonyl reductase of pig lung. Biochem. Biophys. Res. Commun. **194**, 1311–1316

46 Wermuth, B., Maeder-Heinemann, G. and Ernst, E. (1995) Cloning and expression of carbonyl reductase from rat testis. Eur. J. Biochem. **228**, 473–479