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*

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The primary product of the prothoracic glands of last instar larvae of Spodoptera littoralis is 3-dehydroecdysone (3DE). After secretion, 3DE is reduced to ecdysone by 3DE 3β-reductase in the hemolymph. We have previously purified and characterized 3DE 3β-reductase from the hemolymph of S. littoralis. In this study, cDNA clones encoding the enzyme were obtained by reverse transcription-polymerase chain reaction, employing primers based on the amino acid sequences, in conjunction with 5'- and 3'-rapid amplification of cDNA ends. Multiple polyadenylation signals and AT-rich elements were found in the 3'-untranslated region, suggesting that this region may have a role in regulation of expression of the gene. Conceptual translation and amino acid sequence analysis suggest that 3DE 3β-reductase from S. littoralis is a new member of the third superfamily of oxidoreductases. Northern analysis shows that 3DE 3β-reductase mRNA transcripts are widely distributed, but are differentially expressed, in some tissues. The developmental profile of the mRNA revealed that the gene encoding 3DE 3β-reductase is only transcribed in the second half of the last larval instar and that this fluctuation in expression accounts for the change in the enzyme activity during the instar. Southern analysis indicates that the 3DE 3β-reductase is encoded by a single gene, which probably contains at least one intron.

Moulting and many other physiological processes in insects are regulated by molting hormones (ecdysteroids) (1). In immature stages, the prothoracic glands are the primary source of ecdysteroids, generally ecdysone in most species. However, it has become apparent that in most Lepidopteran species studied, the major product of the glands is 3-dehydroecdysone (3DE), together with a varying proportion of ecdysone (2–4). For instance, the glands of Manduca sexta and Pieris brassicae secrete in excess of 95% 3DE, whereas other Lepidopteran species secrete only a 1:1 ratio (Leucinia separate) and others (Bombyx mori) merely traces of 3DE (2–5). Thus, even within a single order, the ratio of ecdysteroids from the prothoracic glands are markedly different. The physiological significance of these differences remains to be explained. Previously, we demonstrated that the prothoracic glands of last instar larvae of Spodoptera littoralis primarily secreted 3DE (~82%), with lesser amounts of ecdysone (~18%) (6). The fact that interconversion of ecdysone and 3DE by prothoracic glands was not detectable suggested that 3DE is more likely an independent product of pathways of ecdysteroid biosynthesis in the glands.

After secretion from the prothoracic glands, the 3DE is reduced to ecdysone by an NAD(P)H-linked 3DE 3β-reductase in the hemolymph (3, 4, 6, 7). Ecdysone undergoes C-20 hydroxylation in certain peripheral tissues yielding 20-hydroxyecdysone, which is considered to be the true molting hormone in most insects (8). Thus, 3DE 3β-reductase-catalyzed reduction of 3DE to ecdysone is viewed as an important regulatory step in the production of the molting hormone in Lepidopteran species. 3DE 3β-reductase was demonstrated in the hemolymph and the activity of the enzyme during the last larval instar reached a peak just preceding that of the hemolymph ecdysteroid titer, supporting a role of the enzyme in production of ecdysteroids. It was revealed that the enzyme exhibited maximum activity at low 3DE substrate concentrations, with a drastic inhibition of activity at higher concentrations (>5 μM), suggesting the existence of an inhibition site for 3DE (6).

The 3β-reductase enzyme was purified from the hemolymph and was shown to be a monomer with molecular mass of approximately 36 kDa (6). Amino acid sequences of the NH2 terminus as well as of interior tryptic peptides of the purified enzyme have been determined. Here, we report the molecular cloning and characterization of the cDNA encoding 3DE 3β-reductase of the cotton leafworm, S. littoralis. Conceptual translation and amino acid sequence analysis indicates that 3DE 3β-reductase is closely related to those of the third superfamily of oxidoreductases.

EXPERIMENTAL PROCEDURES

3DE 3β-Reductase Purification and Protein Sequencing—3DE 3β-reductase from hemolymph of S. littoralis was purified to homogeneity using a combination of polyethylene glycol 6000 precipitation and successive FPLC fractionation on Mono Q, phenyl Superose (twice), and hydroxyapatite columns, as described previously (6). Purified protein was subjected to SDS-PAGE on 10% gels, electrotransferred to ProBlotTM membrane, and visualized by Coomassie staining (9). A single band was observed, excised and sequenced by an automated pulsed liquid-phase sequencer (Applied Biosystems 471A). The NH2-terminal amino acid sequence thus determined was as follows, ATIDVPMLKMGMDNREMPIALGTYLGFDKG. To obtain sequences of the interior region of the enzyme, the purified protein was resolved by SDS-PAGE as described above, visualized by Coomassie staining, and the corresponding band was excised and cleaved with trypsin. The resulting tryptic peptides were purified by high-performance liquid chromatography and
sequenced. The sequences of four of the resulting peptides were determined. These peptides had the following sequences: peptide 1, HFDTA-AIYNTAEGVGEAIR; peptide 2, FGMDLPFGP; peptide 3, LIKEEIEK; peptide 4, INQFNSNTR.

cDNA Cloning and Sequencing—A PCR-based cloning strategy was used to generate cDNA fragment encoding the region between the NH2 terminus and an internal peptide sequenced as described above. Three degenerate primers were synthesized. Primer N was designed on the basis of a part of the NH2-terminal amino acid sequence (5'-GGCAT-TCTYNAAAYGGMNGARAGCG, where Y represents T/C, R is A/G, M is A/C, N is A/T/C/G); reverse primers A and B were made according to the sequences of peptide 1 and peptide 2, respectively, incorporating an EcoRI site (underlined)). An EcoRI restriction site was built into primer N from Spodoptera b. mori (a gift from Dr. A. Mange, Universite Claude Bernard Lyon 1) (11) and an EcoRI site was added to EcoRI and BamHI sites in primer N and a BamHI site in primer A and primer B for use in cloning; these are underlined in the primer sequences.

Since the site of synthesis of the reductase enzyme was unknown, total RNA was extracted using TRIzol (Life Technologies, Inc.) from total insect tissues (including hemolymph, but with the cuticle, head, and gut contents dispersed) dissected from larvae 90 h into the last larval instar. mRNA from total RNA was isolated using Dynabeads mRNA Purification Kit (Dynal) (UK) Ltd. First strand cDNA was reverse transcribed from the mRNA using a 1st Stratum cDNA Synthesis Kit from Boehringer Mannheim with either random primer p(dN)6 and adapter primers Q0 and Q1 (5'-GGCAGCTGCAGGACGTTCAACCCCTAAGGGCGG, respectively, which incorporated an EcoRI site (underlined)) in combination with the following gene-specific primers: 5'-GGCAGCTGCAGGACGTTCAACCCCTAAGGGCGG, respectively, incorporating an EcoRI site (underlined)) in combination with the adapter primers Q0 and Q1, 5'-GGGATCCTAGCTAGACGCT and 5'-TAAGGATCCGCCGACGCT, respectively. Sequencing was performed by the dideoxy termination method using Sequenase Version 2.0 (USBTM, Amersham Pharmacia Biotech). Sequencing was performed by the dideoxy termination method using Sequenase Version 2.0. The DNA sequence containing the entire open reading frame and 3'-noncoding region was amplified by PCR from the cDNA synthesized with primer Q4 as described above using the following gene-specific primers: 5'-GGCAATTCATCTGTGGCGC-CAGTTTT and 5'-GGGATCCTAGCTAGACGCT, respectively. PCR products were purified, digested with EcoRI and PstI and ligated into plasmid pBluescript vector that had been linearized by EcoRI and PstI digestion. Ligation reactions were carried out essentially as described previously (13). Briefly, hemolymph samples taken at various times during the last larval instar were separated by SDS-PAGE on a 10% gel in accordance with the method of Laemmli (14) and electrophoretically transferred to a nitrocellulose membrane (Schleicher and Schuell) (15). Immunoblotting was performed using the antibodies at a final dilution of 1:15,000. Blots were washed at high stringency (0.1 × SSC, 0.1% SDS) and labeled bands visualized by autoradiography.

Preparation of Antibodies and Western Immunoblot Analysis—Polyclonal antibodies against 3DE 3β-reductase were raised in chickens using the purified protein as antigen. The purified protein was first resolved by SDS-PAGE on a 10% gel, visualized with 0.25M KCl. The resulting protein band of apparent molecular mass 36 kDa (see Fig. 4. in Molecular Characterization of 3-Dehydroecdysone 3β-Reductase) was excised from the gel, and mixed with an equal volume of complete Freund’s adjuvant and injected into a chicken. Booster injections were made 12 and 20 days later with 100 μg of the antigen. Antibodies from the yolk of the immunized chickens were polyethylene glycol activated and coupled with protein A and protein G to separate bands visualized by autoradiography.

RESULTS

Cloning of the cDNA Encoding 3DE 3β-Reductase—Using a combination of polyethylene glycol 6000 precipitation and column chromatography on Mono Q, phenyl Superose, and hydroxyapatite, we purified 3DE 3β-reductase to homogeneity from Spodoptera hemolymph. A silver-stained SDS gel revealed the purified 3DE 3β-reductase to be a single band of apparent molecular mass 36 kDa (Fig. 4. in Molecular Characterization of 3-Dehydroecdysone 3β-Reductase). The protein was excised from the gel and subjected to N-terminal amino acid sequence analysis. The N-terminal amino acid sequence of the purified 3DE 3β-reductase was determined by the method of Edman (16). The N-terminal amino acid sequence of the purified 3DE 3β-reductase was determined by the method of Edman (16).
Nh2 terminus and four tryptic peptides derived from purified 3DE double-underlined part that is missing in another clone. The predicted signal peptide is boxed shown in uppercase letters left amino acid numbers are indicated on the indicated in bold, similar to proteins which belong to the third superfamily of GCG package. The cDNA gene product was found to be most all sequences in the data base Swiss-Prot using FASTA in the acid sequence for the cDNA coding region was compared with the Third Superfamily of Oxidoreductases—protein is mildly acidic with an estimated pI of 6.23.

FIG. 1. Nucleotide and deduced amino acid sequences of 3DE β-reductase. Nucleotide numbers are indicated on the right, and amino acid numbers are indicated on the left. The open reading frame is shown in uppercase letters, and the 5′- and 3′-non-coding regions are shown in lowercase letters. The putative polyadenylation signals are indicated in bold, and the sequence indicated in italics identifies the part that is missing in another clone. The predicted signal peptide is double-underlined, and the amino acid sequences obtained from the NH-terminus and four trypptic peptides derived from purified 3DE β-reductase cDNA, whereas

3DE β-reductase particularly resembles mammalian aldose reductases. Among the 20 proteins most closely matched, six are mammalian aldose reductases, others include a mammalian alcohol dehydrogenase, a probable mammalian trans-1,2-dihydrobenzene-1,2-diol dehydrogenase, two mammalian aldose reductase-related proteins, an amphibian lens protein, a putative reductase from Leishmania, a mammalian 3o-hydroxysteroid dehydrogenase, a mammalian chloride-cone reductase, a mammalian trans-1,2-dihydrobenzene-1,2-diol dehydrogenase, a yeast xylose reductase, a mammalian 3-oxy-5-β-steroid 4-dehydrogenase, a plant β-sorbitol-6-phosphate dehydrogenase, a hypothetical 37.1-kDa protein in hxt5-cc12 intergenic region from yeast and a plant 6-deoxycyclanone synthase. The amino acid similarity extends across the entire length of the proteins compared. Fig. 2 shows the comparison of the deduced amino acid sequence of mature 3DE β-reductase to some proteins corresponding to different subgroups of the third superfamily of oxidoreductases. It demonstrates that there is 42.1, 41.3, 39.2, 37.6, and 35.4% identity, respectively, between 3DE β-reductase and human alcohol dehydrogenase, rabbit aldose reductase, rat 3-oxy-5-β-steroid 4-dehydrogenase, rat 3o-hydroxysteroid dehydrogenase, and frog crystallin.

Tissue Distribution and Developmental Expression of 3DE β-Reductase mRNA—As demonstrated in Fig. 3A, a cDNA probe representing the protein coding and 3′-non-coding regions of 3DE β-reductase detected a major transcript of approximately 1.4 kilobases in a variety of tissues prepared from larvae 78 h into the last larval instar. The size of this major transcript is consistent with that of the cDNA obtained. The highest expression of 3DE β-reductase is detected in Malpighian tubules, followed by midgut and fat body, with low levels expressed in hemocyte. No detectable expression was found in the central nervous system (data not shown). Less expression is detected in Malpighian tubules and hemocytes dissected at 46 h into the last larval instar, but no expression was found in all four tissues from early instar larvae. It is noticeable that RNAs at approximately 2.6 and 3.6 kb are also recognized in fat body by this probe but at lower intensity (Fig. 3A). It remains to be determined whether these larger RNAs represent pre-mRNAs for 3DE β-reductase or other mRNAs with significant sequence similarity to β-reductase, although the former seems most probable due to the high stringency used. The signals detected by the actin probe used for normalization are substantially lower in the fat body than those in other tissues (Fig. 3B). RRNA probe was therefore used to further verify the equal loading (Fig. 3C). Some apparent degradation of rRNA is observed in midgut, Malpighian tubules, and hemocytes, which may be due to the fact that 28 S ribosomal RNA in insects frequently occurs as two equal-sized fragments due to RNase activity within certain cells (16). This activity seems least in the fat body and the total amount of hybridization to the rRNA probe seems comparable with the other samples.

Northern analysis of total RNA isolated from whole animals from different developmental stages of the last larval instar revealed that the 3DE β-reductase mRNA is only expressed in the second half of the instar (Fig. 4). The mRNA starts to be detected at 66 h into the last larval instar and increases in intensity, as normalized to actin and rRNA, to 90 h with an apparent decrease at 96 h before increasing to its highest levels observed at 114 h.

Southern Blot Analysis of Genomic DNA—Genomic DNA was prepared from whole animals and digested with various restriction enzymes, of which SalI, HindIII, and NcoI sites are found in the 3β-reductase cDNA, whereas EcoRI and PvuII sites are not found in the cDNA. The resultant DNA fragments
were analyzed by Southern blot using probes representing different regions of the cDNA. As shown in Fig. 5A, usually two bands were observed in all of the digested samples, when the blot was subjected to hybridization with a probe representing the coding and 3'-noncoding regions of the 3β-reductase cDNA. However, when the same blot was re-probed with a cDNA fragment that corresponded to the NH2-terminal region to the first HindIII site in the cDNA, essentially only one band was detected except for the restriction digestion with PvuII, where two bands were observed (Fig. 5B). Again, a probe that represents the region from the SalI site to the poly(A) site detected only one band in each case (Fig. 5C). The analysis displayed in Fig. 5 is most simply interpreted if the 3β-reductase is encoded by a single copy gene in the Spodoptera genome and if this gene has at least one intron that contains EcoRI, PvuII, and SalI sites.

**Fig. 2.** Alignment of the deduced amino acid sequences of mature 3DE 3β-reductase and some database proteins. The deduced mature 3DE 3β-reductase sequence was compared with all sequences in the Swiss-Prot database using the FASTA program in the GCG package. Only the amino acid sequences of some similar proteins representing different subgroups of the third superfamily of oxidoreductases are shown in the alignment. Gaps introduced to maximize alignment are indicated by dots. Identical amino acids are indicated in shaded boxes. Numbers on the right refer to the last amino acid residue in each line of the respective protein sequences. Abbreviations and accession numbers are as follows: ALDX, alcohol dehydrogenase (P14550); ALDR, aldose reductase (P15122); ALDR, 3-oxo-5β-steroid 4-dehydrogenase (P31210); DIDH, 3α-hydroxysteroid dehydrogenase (P23457); CRO, Rho crystallin (P17264). The alignment was constructed by use of the PILEUP program.

**Fig. 3.** Northern blot analysis of the tissue distribution and expression of 3DE 3β-reductase mRNA. 10 μg of total RNA from various tissues (top) at different times (bottom) within the last larval instar was used. The blots were hybridized with a 32P-labeled 3DE 3β-reductase cDNA probe containing the protein coding and 3'-noncoding regions (A), followed by stripping and re-probing with actin (B) and rRNA (C) probes, respectively, as indicated on the right side of each panel. Northern blot signals were quantified using a Molecular Dynamics densitometer, and values (with ranges) of two separate experiments were normalized with actin (open column) and rRNA (shaded column) respectively (D). The dashed line illustrates the fluctuation of the enzyme activity during the instar.
amino acid sequence of the cDNA contained the NH2-terminal coding the enzyme was isolated and sequenced. The predicted top digested with various restriction enzymes as indicated on the left panel. The blot was hybridized with 32P-labeled cDNA corresponding to: the entire coding region and 3'-noncoding region (bases 1–1386 in Fig. 1A), a region from the start codon to the first HindIII site (bases 1–319) (B), and a region from the SstI site to the 3'-end (bases 809–1386) (C). The sizes of DNA marker are indicated on the left.

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 3β-reductase, together with 3’- and 5’-RACE, the complete cDNA encoding the enzyme was isolated and sequenced. The predicted amino acid sequence of the cDNA contained the NH2-terminal sequence of the enzyme as well as the four internal peptides obtained from tryptic digestion of the band corresponding to the 36,000 polypeptide, confirming that we had cloned the 3DE 3β-reductase cDNA. The presence of 3DE 3β-reductase enzyme in hemolymph is due to secretion from tissues that synthesize it. The first 17 predicted amino acids appear to constitute a signal peptide, since the sequence shares significant characteristics with the signal sequences of some eukaryotic secretory proteins (17): (i) the 17 amino acids are within the typical range of 13–36 residues of signal peptides; (ii) a positively charged residue, arginine, is found in the amino-terminal part of the signal; (iii) it contains a highly hydrophobic stretch of 11 residues; (iv) a serine residue at the end of the signal has a small neutral side chain that is recognized by signal peptidase for cleavage.

Three putative polyadenylation signals were found in the 3’-noncoding region of the cDNA, suggesting alternative polyadenylation sites may exist, as the cleavage and polyadenylation specificity factor may bind to any one of these three sequences (18). Indeed, during sequencing following 3’-RACE, we found that one cDNA clone showed a shorter 3’-noncoding region, with the poly(A) tail attaching 14 nucleotides downstream of the second AATAAA sequence (Fig. 1). Furthermore, three ATTTA sequences were found in the 3’untranslated region (3’-UTR) of the 3β-reductase cDNA, all located in between the second and the third polyadenylation signals, with two occurring in the sequence which is missing in the shorter 3β-reductase cDNA (Fig. 1). In fact, the 3’-untranslated region has been increasingly recognized as one of the important sequences which may contain distinct elements involved in regulating mRNA degradation (19). A most well studied example is the AU-rich element (ARE) found on many unstable mRNAs. The sequence consensus for the AREs is loosely defined as AUUUA repeated once or several times within the 3’-UTR. This pentanucleotide is often found within an AU-rich region of the mRNA. It seems to be clear that (i) both nuclear and cytoplasmic proteins can specifically bind to the ARE, (ii) the binding activity of some of those proteins appears to increase or decrease in response to the changes in cellular metabolism that lead to alterations in the stability of ARE-containing mRNA, and (iii) ARE activity can be mediated by a complex of proteins as in the case of a 20 S complex (19). We were interested in addressing the question as to whether the alternative polyadenylation, using AATAAA signals at different locations in the 3β-reductase cDNA, is related to the regulation of mRNA stability and what the role of the AREs in the 3’-UTR is likely to be. For this, total RNA from fat body, midgut, and Malpighian tubules from 78 h last larval instar larvae was subjected to Northern analysis using probes corresponding, respectively, to nucleotides 1072–1176, 1177–1281, and 1282–1386 of the cDNA (see Fig. 1). Because all three probes gave identical signals, the AREs in the 3’-UTR do not seem to contribute to the regulation of the mRNA stability in the tissues examined (data not shown).

It has long been recognized that oxidoreductases, dependent upon nicotinamide coenzymes, can be divided into two extended superfamilies, according to their structural type and catalytic mechanism (20). The enzymes of one superfamily contain catalytically important zinc, many utilizing primary alcohols as substrates, though use of secondary alcohols and other activities are also noticed (21–24). A structurally noteworthy feature of these proteins is the highly conserved cysteine residues, involved in zinc binding, scattered within the
The predicted amino acid sequence of the mature protein indicated one cysteine residue (in position 192, see Fig. 1) in the pre-interconverting ecdysone and 3DE (ecdysone oxidase and 3DE proteins). Hence, we conclude that 3DE has a wide tissue distribution with considerable variability in such tissue 3DE. The Northern analysis has revealed that the mRNA for 3DE is a new member of the third superfamily of oxido-reductases.

Northern analysis revealed that the mRNA for 3DE-reductase has a wide tissue distribution with considerable variability in the abundance of the mRNA among the tissues examined. It is not clear at present which tissue is the major source for the 3β-reductase activity in the hemolymph or whether all the tissues containing 3β-reductase mRNA contribute to the enzyme activity in hemolymph. The cytosolic enzymes reversibly interconverting ecdysone and 3DE (ecdysone oxidase and 3DE-reductase) have been reported to be widely distributed, even in tissues that do not reduce 3DE to 3-epiecdysone (31, 32). If such tissue 3DE 3β-reductase is closely related to the hemolymph 3β-reductase, the wide distribution of transcripts in the Northern blots could be explained by hybridization of the probe to mRNA corresponding to such tissue 3β-reductase. Sakurai and co-workers (33) examined the tissue distribution of the 3β-reductase in B. mori at the protein level, as well as the level of enzyme activity, and they too found a wide tissue distribution. The absence of the 3β-reductase protein in central nervous system in B. mori is consistent with our observation that 3β-reductase mRNA is not detectable in that tissue from S. littoralis. However, the fact that Malpighian tubules and midgut from B. mori do not contain detectable enzyme protein seems to be surprising, since we found that the mRNA for 3β-reductase is abundantly transcribed in these tissues from S. littoralis. Preliminary results of Western blotting with tissues dissected from S. littoralis at 90 h into the last instar revealed a similar distribution pattern as that of B. mori, with 3β-reductase being detected in hemolymph as well as hemocytes, but little in midgut and none in Malpighian tubules (data not shown). It seems, therefore, that although the mRNA is abundantly transcribed in Malpighian tubules and midgut, none or only little is translated into the protein in these tissues. This observation may indicate that selective translation of mRNA is indeed an important step in regulation of expression of the gene encoding 3DE 3β-reductase.

Northern analysis also revealed that the mRNA for the enzyme is only expressed in the second half of the last larval instar. The mRNA starts to be detected 48–66 h into the last larval instar, at which time the actual enzyme activity is just about to appear (6). The increase in expression can be seen from 72 to 90 h, which coincides well with the sharp rise in the enzyme activity. The small decline in the expression between 90 and 96 h is also coincident with the decrease in the enzyme activity at this time. The second increase in the mRNA level observed between 102 to 114 h is presumably responsible for the small rise in the enzyme activity at the end of the instar. It appears evident, therefore, that the change in the activity of 3β-reductase is, at least in part, regulated by gene expression at the transcriptional level, although, as mentioned above, post-transcriptional regulation may also be as important in the expression of 3DE 3β-reductase in certain tissues.

Southern analysis suggest that 3β-reductase is encoded by a single copy gene in the genome of S. littoralis. Although the details of the organization of the gene are far from clear, it is predicted from the results of Southern analysis that the gene encoding 3β-reductase has at least one intron that contains EcoRI, SalI, and PvuII sites. It remains to be determined whether the existence of two larger RNAs detected in fat body by a probe representing the entire coding region and 3′-non-coding region of the cDNA is due to the possible occurrence of intermediates in the process of mRNA splicing.

The availability of the cDNA clones encoding 3DE 3β-reductase should facilitate studies of genomic organization, regulation of expression of the gene, structure-function relationships of the enzyme, and its involvement in regulation of the production of ecdysteroids.

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