Molecular Structure of Rat Hepatic 3α-Hydroxysteroid Dehydrogenase
A MEMBER OF THE OXIDOREDUCTASE GENE FAMILY*

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3-α-Hydroxysteroid dehydrogenase (3α-HSD) (EC 1.1.1.50) is an important multifunctional oxidoreductase capable of metabolizing steroid hormones, polycyclic aromatic hydrocarbons, and prostaglandins. 3α-HSD is also required for bile acid synthesis and has been suggested to play an important role in net bile acid transport across the hepatocyte (Stolz, A., Takikawa, H., Ookhtens, M., and Kaplowitz, N. (1989) Annu. Rev. Physiol. 51, 166–177). In order to characterize molecular forms and begin to determine its regulation, we now report the nucleotide sequence, tissue distribution, and homology to other members of the oxidoreductase superfamily. Rat hepatic 3α-HSD cDNA encodes for a 322-amino acid protein with a predicted molecular weight of 37,022 expressed in a 2.4-kilobase (kb) message. Northern blot analysis of total RNA revealed equivalent steady-state levels in liver and intestine in male rats with lower levels of expression in the colon and minimal expression in stomach, lung, and testis. Female liver contained approximately 2–3-fold greater steady-state levels of mRNA as compared to the male liver with equivalent intestinal expression. Two hybridizing bands, 2.4 and 1.4 kb, were identified in total RNA from the ovary. 3α-HSD exhibits 75% amino acid sequence homology with bovine lung prostaglandin F synthetase and 50% homology with human aldose reductases. Amino acid sequence analysis with short chain alcohol dehydrogenases identified a possible NADP(H) cofactor-binding site at the amino terminus. The significant homology of 3α-HSD with both prostaglandin F synthetase and aldose reductases suggest a subdivision of monomeric, NADPH reductases within the larger oxidoreductase superfamilies.

3-α-Hydroxysteroid dehydrogenase (3α-HSD)1 (EC 1.1.1.50) is an important, multifunctional reductase which metabolizes steroid hormones and polycyclic aromatic hydrocarbon carcinogens (1, 2). 3α-HSD catalyzes the stereospecific reduction of cortisol, progesterone, and testosterone by preferentially utilizing the cofactor NADPH. Reduction of these hormones is rapidly followed by conjugation at either the 3 or 17 position with sulfates or glucuronides leading to their ultimate elimination. In addition to its role in steroid hormone metabolism, 3α-HSD serves a dual function in rat liver being required for both bile acid synthesis and the efficient intercellular transport of bile acids (3–7). 3α-HSD stereospecifically reduces the bile acid precursors, 7α,5β-cholestan-3-one and 7α,12α-dihydroxy-5β-cholestan-3-one formed during the synthesis of the primary bile acids. We have previously demonstrated that bile acids binding to the cytosolic 3α-HSD plays an important role in efficient intercellular transport of bile acids from the sinusoidal to the canalicular pole of the rat hepatocyte (4).

In rat liver, 3α-HSD metabolizes prostaglandins and copurifies with dihydrodiol dehydrogenase which is capable of metabolizing polycyclic aromatic hydrocarbon carcinogens (2, 8–10). This enzyme is competitively inhibited by the nonsteroidal class of anti-inflammatory agents such as indomethacin, further suggesting a possible role in the inflammation response (1, 11). In rat and other species, multiple forms of dihydrodiol dehydrogenase activities exist, some of which copurify with the 3α-HSD. Dissociation of 3α-HSD activity from dihydrodiol dehydrogenase activity is both organ- and species-dependent (12–15).

Overlapping substrate specificities of the 3α-HSD with other reductases and purification of only a few forms has hindered precise knowledge about isoforms, detailed mechanism of enzymatic catalysis, and gene regulation. In order to further characterize the rat hepatic 3α-HSD at both the structural level and begin to analyze its regulation, we have identified and sequenced the rat hepatic 3α-HSD cDNA. This cDNA sequence encodes for a protein of 322 amino acids with a molecular mass of 37,022 daltons contained in a 2.4-kb message. We now report the deduced cDNA sequence, tissue distribution, and significant homology to other members of the oxidoreductase superfamily.

MATERIALS AND METHODS AND RESULTS*

DISCUSSION

The organ distribution of 3α-HSD mRNA expression concurs with the previously determined immunoreactivity for the

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1 The abbreviations used are: 3α-HSD, 3α-hydroxy-steroid dehydrogenase; kb, kilobase(s).

2 Portions of this paper (including "Materials and Methods," "Results," Figs. 1, 2, 4–7, and Table 1) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
copurified Y' bile acid binder and for indomethacin inhibitable 3α-HSD activity (Fig. 4) (44, 45). In contrast, rat dihydrodiol dehydrogenase activity is greatest in the liver and lung followed by heart and intestine (45). Only liver and intestinal dihydrodiol dehydrogenase activity is significantly inhibited by 6-medroxyprogesterone acetate suggesting that the intestinal enzyme is eluted by the hepatic form of the 3α-HSD. Our finding of significant hepatic 3α-HSD mRNA in the small intestine is consistent with these prior findings. Purification and sequence analysis of rat dihydrodiol dehydrogenase activity in non-hepatic and intestine tissues will be required to determine the precise relationship between the hepatic 3α-HSD and dihydrodiol dehydrogenase activity in these other tissues. The predominant localization of 3α-HSD in the liver and intestine suggests a role in xenobiotic metabolism.

The localization of 3α-HSD activity in rat brain and ovary suggests an important role in the metabolism of progesterone and dihydrotestosterone. 3α-HSD activity has also been identified in non-neuronal cells of the olfactory tubercles, pituitary cytosol, and has been purified to homogeneity from total rat brain (46-49). This purified enzyme is potently inhibited by 6-medroxyprogesterone as is the hepatic 3α-HSD activity. In the pituitary, 3α-HSD has been implicated in regulation of both ovulation and gonadotropins by reduction of 5α-pregnane-3,20-dione. The speculated role of the 3α-HSD in the olfactory tubercle is to metabolize dihydrotestosterone which may mediate gonadotropin-releasing hormone release. The failure to identify 3α-HSD message in total brain RNA may be due to either low or localized levels of 3α-HSD mRNA or that the 3α-HSD activity reflects the product of another gene.

Ovarian 3α-HSD activity has been implicated in playing an important role in ovarian follicular development by its capacity to metabolize androgens. Dihydrotestosterone inhibits aromatase activity which is critical for follicular development. Ontogeny studies and selective irradiation of granulosa cells implicating the granulosa as cells of the site of 3α-HSD activity (50-52). Ovarian 3α-HSD activity fluctuates with estrus and is regulated by gonadotrophins or gonadotrophin-induced steroids indicating a complex mode of regulation. Studies are presently underway to define the cellular localization of the 3α-HSD and to determine the significance of the 1.4-kb hybridizing mRNA.

3α-HSD exhibits striking amino acid homology to both bovine lung prostaglandin F synthetase and human aldose reductases. All these proteins are members of the aldo-keto reductase superfamily, a large family of proteins capable of reducing carbonyl groups on a wide variety of compounds, including sugars, steroid hormones, ketones, and xenobiotic aldehydes. Prostaglandin F synthetase is capable of metabolizing some of the same xenobiotics as the rat hepatic 3α-HSD, but inefficiently reduces dihydrotestosterone (35). The competitive inhibition of 3α-HSD by indomethacin and its capacity to oxidize hydroxyprostaglandins confirms the close homology between these two proteins. Studies are being performed to identify the granulosa cells as the site of 3α-HSD activity (50-52). Ovarian 3α-HSD activity fluctuates with estrus and is regulated by gonadotrophins or gonadotrophin-induced steroids indicating a complex mode of regulation. Studies are presently underway to define the cellular localization of the 3α-HSD and to determine the significance of the 1.4-kb hybridizing mRNA.

In conclusion, we have identified the cDNA for the rat hepatic 3α-HSD. The predominant location of the 3α-HSD in the liver and intestine is consistent with its role in xenobiotic metabolism and participation in the intracellular transport of bile acids. Future studies will require the cDNA analysis of other isoforms of 3α-HSD and dihydrodiol dehydrogenase activity to determine their relationship among themselves and to both the dimeric short/long chain alcohol/dehydrogenase activity. The cDNA analysis of these various genes and may provide insight into mechanism of gene evolution in these large protein superfamilies.

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Supplemental Material

Molecular Structure of Rat Hepatic 3α Hydroxysteroid Dehydrogenase

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Molecular and Cell Biology lab.

Chemistry

Immunohistochemistry

DNA Cloning

Both a commercial female rat liver cDNA library (RL.100b) [Chromon, Palo Alto, CA] and a female Sprague-Dawley rat liver gel band (pH 7.0) library kindly provided by Dr. C. Clarke and P. Edwards (UCLA) were utilized (20). 800,000 p.f.u. were screened and duplicate nitrocellulose filters probed as described by Maniatis (21). Nitrocellulose filters [Hybond-N+, Schleicher & Schuell, Keene, NH] were probed for two hours in 5 X Denhardt's, (1 X Denhardt's = 0.2% Ficoll 400, 0.2% polyvinylpyrrolidone and 5% bovine serum albumin) 50 mM sodium citrate, 0.5 M sodium chloride, 0.05 M sodium phosphate, pH 7.4, 2 mM EDTA at 37°C and a 0.5% SDS wash in 50 mM sodium phosphate, 0.1% SDS, 2 mM EDTA at 37°C followed by 0.5% SDS washes at 37°C and 0.05% SDS at 23°C for 30 minutes. Filters were then exposed to Kodak XAR film and intensifying screen at 80°C for 16 to 48 hours and corresponded clones plucked from the film. A second library was screened with an oligonucleotide (5'-GAA-AAT-CTY-CCT-GAA-CAG-ATC-ACG) corresponding to the 5' end of the cDNA in a full length clone. Over 100 clones were identified on primary screening of 32,000 p.f.u. 36 clones were plate purified and amplified via the polyethylene glycol reaction for evaluation of internal restriction using the following primers pair: (a) lambda g3 (specific sequence from lambda directed primer towards the 5' end with 3' lambda primer and (b) internal primer directed towards the 5' end with 3' lambda primer (23)). The DNA was digested by restriction endonuclease digestion (Eco RI). Individual fragments were separated into plasmid vector, pSP79 (Pharmacia, Piscataway, NJ) and underlaid RNA/DNA sequence analysis with dATP digoxigenin (Amersham, Arlington Heights, IL) using Sequence 2.07 Tm+ polymerase (United States Biochemical Corp., Cleveland, OH) (24). Synthetized oligonucleotide primers corresponding to the DNA were used to generate overlapping sequences in both orientations.

Northern Analysis

Total cellular RNA from male and female rat organs were isolated according to the method of Chomczynski et al. and quantified by 260 nm absorbance (25). 10 μg of total RNA was hybridized, reannealed in 2 μL of 2× DEPC treated water and 0.5 μL of RNA sample buffer (1× MOPS = 20 mM MOPS (N-Morpholinososuberic acid), 5 mM sodium acetate, 1 mM EDTA, pH 7.0, 2.5 M formaldehyde, 0.5% formamide) and 2 μL of ethidium bromide (100 μg/mL) (0.1% DEPC treated water). The sample was then heated for 5 minutes at 70°C, allowed to cool slowly to 37°C and added 3 μL of lysis buffer containing 1 μL of MOPS, 0.6% formaldehyde. Gel were washed in 2× SSC for 1 hour and then transferred onto Nytran (Schleicher & Schuell), Kent, NH, USA by capillary action with 20× SSC, then subjected to hybridization with a denatured biotin labeled 0.5 kb insert (20). Membranes were hybridized according to the method of Church and Gilbert (27).

Southern Analysis

In vivo, 7, 1 and 0.5 μg of total RNA from male and female liver, intestine and colon, isolated as described above, were electroblotted onto Nytran membrane, hybridized as described above and exposed to Kodak XAR film at -30 °C using intensifying screens. Blots were quantified by densitometric analysis using an LKB densitometer (Pharmacia, Piscataway, NJ).

Ligand Binding Assay

All biochemistry studies analysis was performed on the University of Wisconsin GCG computer program utilizing GENEBANK version 62 due to a VAX microcomputer.

Results:

cDNA cloning: DNA cloning of the 3α-HSD gene was dependent on amino acid sequence analysis for both aligning oligonucleotide probes to rat cDNA libraries and for verification of the isolated cDNA. Figure 1 illustrates one of the two 3α-HSD peptide maps. Five peptide fragments were expected from the peptide map and sequenced for these objectives. The initial cDNA cloning of the rat hepatic 3α-HSD was performed initially on the peptide probe based on peptide sequence data which was 87% identical to the actual cDNA sequence. Figure 2 illustrates the oligonucleotide probe used for initial library screening, sequencing map and cloning strategy utilized for the DNA sequencing. Figure 3 contains the nucleotide sequence with its deduced amino acid sequence. The 3α-HSD terminal is clearly identified by the presence of two stop codons in frame immediately preceding a spars of 19 amino acid deduced by protein microsequencing (SAB-3 and SAB-4, respectively), followed by a single amino acid insertion. During multiple attempts, no amino residues amino sequence was obtained. These three lines evidence support the location of the initial microsequence in Figure 3. Five potential microsequence origins in the rat cDNA were identified. The six nucleotide sequence corresponded to the 3α-HSD gene. The RNA message size of 2.4 kb also agrees with the cDNA clone identified.
Figure 4: Tissue Distribution of Hepatic 3α-HSD gene expression in Male and Female Rats

Total RNA was extracted from male (left panel) and female (right panel) from three Sprague-Dawley rats using the method of Chomczynski (25). 10 μg of total RNA was transferred onto Hybond membranes and probed with 32P labelled proximal Eco R1 fragment of the cDNA (32A) according to the method of Church and Gilbert (27). RNA molecular weight standards in kb are listed on the left of the blot. Equivalent amounts of total RNA were applied as determined by ethidium bromide staining.

Figure 5: Slot Blot Analysis of 3α-HSD steady-state mRNA levels in Male and Female Liver and Intestine

Varying amounts of total RNA from both female and male liver and intestines divided into Proximal Intestine (P.I.), Mid Intestine (M.I.) and Distal Intestine (D.I.) were applied onto a Nytran membrane and probed as in Figure 4. Hybridization data was calculated by densitometric analysis of different RNA amounts. Results are expressed in μg of RNA with standard deviation expressed in arbitrary units of absorbance per μg of RNA.

Homology Studies:

Nucleotide and amino acid homology comparison was conducted utilizing the FASTA algorithm of Pearson to explore the relationship of the 3α-HSD to other oxidoreductases (26). Significant homology was found to Bovine lung prostaglandin F synthetase (IC 11.1.1.18) (PGFS) and Human aldose reductases (EC 1.1.1.21) (ALDR), two other NADPH dependent, monomeric carbonyl reductases, and in epoxide-crystalline proteins of the European common frog (31-34). 70% amino acid identity was found with Bovine lung prostaglandin F synthetase, an enzyme capable of reducing PGF2α to PGF2a via endoperoxidase activity and converting PGES to 9α,11β-PGF2α via 11-keno reductase activity (35). Watanabe has previously demonstrated that rat prostaglandin F synthetase activity is maximal in cytosol of lung, followed by decreased levels in stomach and heart with less activity in kidney and liver (36). This pattern is markedly different than hepatic 3α-HSD gene expression. Human aldose reductases shares 47% amino acid identity with 3α-HSD protein. Figure 6 illustrates the alignment, homology and consensus sequence of these two reductases with the newly identified 3α-HSD. Significant homology is apparent in all three proteins, 3α-HSD also shares 50% homology with the partially sequenced epoxide crystalline lens proteins from common frog (data not shown). Recruitment of soluble oxidoreductases as components of tissue-specific crystallines of vertebrates and invertebrates has been well described (37,38). The relationship of epoxide-crystalline to PGFS and ALDR has also been noted by other investigators (31,33,34).

Comparison of this subgroup of closely related carbonyl reductase to the larger family of alcohol/polyol dehydrogenase allows one to identify possible NAD(P)H cofactor binding sites and conserved, presumed active site sequences. Studies by Joernvall and others have demonstrated the importance of the conservation of glycine residues in maintaining the tertiary conformation of proteins from this large alcohol/polyol dehydrogenase gene family (39,40). The conserved sequence XXXXXXXXXG with variation on this motif has been identified with the NAD nucleotide binding fold described by Rowan (39,41,42). This motif is present in all three carbonyl reductases and for 3α-HSD is at amino acid position 13-22, suggesting that the NADPH cofactor binding site is localized at the amino terminus. All glycines and their position in the 3α-HSD are conserved in the PGFS protein and 11 of 14 glycine positions in ALDR further supporting their close structural relationship.

In addition to the identification of the nucleotide binding fold, 3α-HSD shares a conserved pentapeptide sequences identified by Joernvall in members of the short chain alcohol dehydrogenase family and 15-hydroxyprostaglandin dehydrogenase (43). Figure 7 illustrates this evolutionary conserved sequence Tyr Cys Ala Ser Lys among various short chain alcohol/polyol dehydrogenases. Tyrins in the most conserved element of this sequence pattern is maintained in 3α-HSD except for a single modification of a lysine for alanine at position number 207. This highly conserved sequence is presumed to play an important role in catalytic function which can be best addressed with crystallographic analysis and site directed mutagenesis studies.