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HOMOLOGIES BETWEEN ENZYMES INVOLVED IN STEROID AND XENOBIOTIC CARBONYL REDUCTION IN VERTEBRATES, INVERTEBRATES AND PROCARYONTS

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Summary—Evidence is reported for the existence of a structurally and functionally related and probably evolutionarily conserved class of membrane-bound liver carbonyl reductases/hydroxysteroid dehydrogenases involved in steroid and xenobiotic carbonyl metabolism. Carbonyl reduction was investigated in liver microsomes of 8 vertebrate species, as well as in insect larvae total homogenate and in purified 3α-hydroxysteroid dehydrogenase preparations of the procaryont *Pseudomonas testosteroni*,† using the ketone compound 2-methyl-1,2 di-(3-pyridyl)-1-propanone (metyrapone) as substrate. The enzyme activities involved in the metyrapone metabolism were screened for their sensitivity to several steroids as inhibitors. In all fractions tested, steroids of the androstane or pregnane class strongly inhibited xenobiotic carbonyl reduction, whereas only in the insect and procaryotic species could ecdysteroids inhibit this reaction.

Immunoblot analysis with antibodies against the respective microsomal mouse liver metyrapone reductase revealed strong crossreactions in all fractions tested, even in those of the insect and the procaryont. A similar crossreaction pattern was achieved when the same fractions were incubated with antibodies against 3α-hydroxysteroid dehydrogenase from *Pseudomonas testosteroni*. The mutual immunoreactivity of the antibody species against proteins from vertebrate liver microsomes, insects and procaryonts suggests the existence of structural homologies within these carbonyl reducing enzymes. This is further confirmed by limited proteolysis of purified microsomal mouse liver carbonyl reductase and subsequent analysis of the peptide fragments with antibodies specifically purified by immunoreactivity against this respective crossreactive antigen. These immunoblot experiments revealed a 22 kDa peptide fragment which was commonly recognized by all antibodies and which might represent a conserved domain of the enzyme.

INTRODUCTION

Carbonyl reduction of the 3-oxo group of steroid hormones in vertebrates is catalyzed by 3α-hydroxysteroid dehydrogenase (E.C. 1.1.1.50) and 3β-hydroxysteroid dehydrogenase (E.C. 1.1.1.51). The reaction generally leads to a decrease of hormonal activity and prepares the steroid for conjugation with sulfate or glucuronic acid.

The proteins involved in this biotransformation step constitute a heterogenous group of enzymes with a distribution pattern specific for species, tissue, sex and developmental stage. The enzymes belong to the oxidoreductase class and appear in the cytosolic and microsomal fractions of the cell.

Recent efforts resulted in cloning and sequencing of the 3α-hydroxysteroid dehydrogenase from rat liver cytosol [3–5]. This enzyme is known to also possess 15-keto prostaglandin reductase activity, bile acid binding and *trans* benzene dihydrodiol dehydrogenase activity, the latter being an inactivation step of ultimate

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†Although being classified as *Comamonas testosteroni* [1] the classification *Pseudomonas testosteroni* is used [2].

Abbreviations: MLMR: microsomal mouse liver metyrapone reductase; HSD 28: 3α-hydroxysteroid dehydrogenase from *Pseudomonas testosteroni* with M_w of 28 kDa; kDa: kilo dalton; delta-1-dehydrogenase: 3-oxo-delta-1-hydroxysteroid dehydrogenase (E.C. 1.3.99.4); IEF: isoelectric focusing; NBT: nitro blue tetrazolium; BCIP: bromo-chloro-indolyl phosphate; CM cellulose: carboxymethylcellulose; TBST: Tris buffered saline, 0.05% Tween 20.

carcinogens derived from polycyclic aromatic hydrocarbon metabolism [6]. Sequence similarities were found to members of the aldo-keto reductase superfamily namely prostaglandin F synthetase, aldose reductase, aldehyde reductase and frog lens crystallin. No striking homology could be established to other steroid dehydrogenases, neither of eucaryotic nor procaryotic origin.

Major sites of biotransformation and bioactivation in the liver of vertebrates are the microsomes. While much is known about the oxidative cytochrome *P*450 systems little is known about reductive enzymes of the microsomal fraction. The present knowledge comprises catalytical and functional data derived only from crude fractions or purified proteins mostly from rodents [7–11]. The proteins are considered to be specific for the 3-oxo group of the steroid nucleus. Moreover they often exert enzymatic (i.e. carbonyl reductase) activity towards other endogenous or xenobiotic compounds [7–9].

First insights into the relationship between xenobiotic and steroid carbonyl reduction were achieved with purified enzymes from rat and guinea pig liver microsomes by Sawada and co-workers [7–9], who showed that 3α-hydroxysteroid dehydrogenase could reduce the 3-oxo group of the steroid as well as the keto group of several xenobiotic compounds, e.g. metyrapone to their respective alcohol metabolites. Functional and structural homologies were found by comparison of microsomal liver carbonyl reductases of three rodent species or human liver microsomes [12, 13].

To our knowledge no data on nucleotide or amino acid sequences of mammalian microsomal enzymes involved in reductive carbonyl metabolism of steroids and xenobiotic carbonyl compounds are available to date.

A number of procaryotic species e.g. some *Pseudomonads* can utilize, in contrast to mammalian species, steroids as a sole source of carbon. This indicates that they can degrade steroids and have the respective enzymes. During growth on steroid-containing media a variety of steroid transforming enzymes are induced in *Pseudomonas testosteroni*, e.g. several 3α-hydroxysteroid dehydrogenases, 3β(17β)-hydroxysteroid dehydrogenases, delta 4,5 isomerase and delta 1-dehydrogenase [2, 14, 15]. This induced phenotype might contribute to the ability to live in areas heavily polluted with aromatic and alicyclic hydrocarbons [16].

Previously we reported the immunological and functional similarity between a 3α-hydroxysteroid dehydrogenase from *Pseudomonas spec.* (HSD 28) and microsomal mouse liver carbonyl reductase (mouse liver metyrapone reductase = MLMR) [17]. The intention of this study was to evaluate and confirm the functional and structural related features within this class of enzymes in different phylogenetic species. In this paper we describe the ability and extent of some vertebrate species for microsomal liver carbonyl reduction of a xenobiotic substrate [2-methyl-1,2-di-(3-pyridyl)-1-propanone (metyrapone)], commonly used as diagnostic cytochrome *P*450 inhibitor. We also investigated the inhibition of this model carbonyl reduction by several steroids. The enzyme system is compared with respective reactions in a procaryont (*Pseudomonas spec.*) and an insect species (*Calliphora vicina*) (blowfly).

Information on structural homologies on the protein level was gained by comparing the respective fractions in immunoblot experiments with antibodies raised against a purified procaryotic 3α-hydroxysteroid dehydrogenase (HSD 28) from *Pseudomonas spec.* and antibodies against a purified mammalian carbonyl reductase derived from MLMR. Limited proteolysis and subsequent Western blotting of purified MLMR fragments with affinity purified antibodies against crossreactive proteins were performed to search for a conserved region in the mammalian enzyme.

EXPERIMENTAL

Materials

3α-Hydroxysteroid dehydrogenase (E.C. 1.1.1.50) was purchased from Boehringer Mannheim (Germany) or Sigma (Deisenhofen, Germany). 3α- and 3β-hydroxy desogestrel were from Organon (Oss, The Netherlands), metyrapone from Fluka (Buchs, Switzerland); its alcohol metabolite metyrapol was a kind gift from G. F. Kahl (Department of Pharmacology, University of Göttingen, Germany). All other chemicals employed were from commercial suppliers and of highest analytical grade.

Livers of 4- to 8-month-old NMRI mice, Wistar rats, Hartley guinea pigs and common house chicken of both sexes were prepared from animals of our own laboratory. Livers from sheep, pig and rabbit were obtained from local slaughteries. Human liver samples were from

transplant donors. The consents of the donors' relatives were given and ethical approval was granted.

Preparation of insects

Larvae of the blowfly (*Calliphora vicina*) were grown on beef muscle at 23°C and 50% relative humidity with light from 7 a.m. to 7 p.m. Their developmental age is expressed in days after egg-laying. Homogenizations were carried out by use of an Ultra-Turrax device (Janke & Kunkel, Staufen, Germany) in Ringer solution at 4°C. Brain ring gland complexes were prepared under a stereo microscope and then homogenized in Ringer solution [18]. The resulting homogenate was centrifuged briefly to sediment insoluble material. No antioxidant protection against phenol oxidase was used.

Preparation of liver microsomes

Livers, where possible, were perfused *in situ* with 1.15% icecold KCl solution and homogenized with 4 parts of homogenization buffer (15 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, pH 7.4) with a Potter Elvehjem homogenizer. Cytosolic and microsomal fractions were prepared by standard methods.

SDS–PAGE

SDS–PAGE of microsomal and cytosolic proteins was performed in 10% separating and 4% stacking gels [19]. Protease generated peptides were electrophoresed by use of a tricine based buffer system [20] with a 17% acrylamide separating gel.

Western blot

Immobilizaton of electrophoretically separated proteins on nitrocellulose (Schleicher & Schuell, Dassel, Germany) was achieved performing the semi dry blot technique [21]. Proteins from 10% gels were transferred for 30 min with 0.8 mA/cm^2, and peptides from 17% gels 20 min with 0.5 mA/cm^2, followed by 60 min with 0.8 mA/cm^2. Saturation of unspecific binding sites was achieved with 1% bovine serum albumin (BSA), 5% fetal calf serum (FCS) in 1 × TBST (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20). First antibody incubation was performed overnight, followed by secondary antibody incubation for 2 h and washing steps with 1 × TBST, color development with NBT, BCIP in case of alkaline phosphatase coupled secondary antibodies [22] or chloronaphthol–H_2O_2 in case of peroxidase labeled secondary antibodies [23].

Purification of MLMR and immunization

MLMR was purified as described previously [10, 12]. Immunization of a female rabbit was carried out with 4 × 150 μg purified denatured MLMR and resulted in a high antibody titer antiserum (i.e. a signal could be detected in Western blot with 1 μg of total microsomal protein and an antibody dilution of 1:10,000) against the antigen, the IgG fraction of which was further purified by Protein A Sepharose chromatography (Pharmacia LKB, Freiburg, Germany) as described previously [12].

Purificaton of 3α-OH-dehydrogenase and immunization

Crude fractions of commercially available 3α-hydroxysteroid dehydrogenase from *Pseudomonas spec.* (Boehringer Mannheim) or *Pseudomonas testosteroni* ATCC 11996 (Sigma) were purified after the following scheme, adopted from a method described by Roe and Kaplan [24]: lyophilized enzyme fractions were dissolved in 200 μl 10 mM sodium phosphate buffer, pH 6.2, and incubated for 10 min with 1 ml of CM cellulose prepared after the manufacturer's recommendations and equilibrated in the same buffer. The batch was shortly centrifuged and the supernatant was loaded on a 1.5 mm polyacrylamide gel which was prepared as described under SDS–PAGE except that SDS was omitted in all solutions required. Electrophoresis was performed at 4°C with 180 V constant voltage. After completion border segments of the gel were cut off and stained for 3α-hydroxysteroid dehydrogenase and metyrapone reductase activity [17]. Within these segments two bands of enzyme activity could be detected. The lower one, which corresponds to a protein of 28 kDa in SDS–PAGE was cut out of the gel, homogenized in a microcup and suspended in 50 mM sodium phosphate buffer, pH 7.4. After 2 days at 4°C the slurry was transferred into a new tube perforated at the bottom with a canule and filled with glass wool. The enzyme containing supernatant was centrifuged through the glass wool into a fresh tube. The resulting enzyme preparation was checked for homogeneity by SDS–PAGE and further used for enzyme assays, Western blotting, IEF and N-terminal amino acid sequence determination. SDS–PAGE revealed one single

protein band with a M_w of about 28 kDa, a pI in IEF of 4.7 and one single amino acid sequence when subjected to Edman degradation in a gas phase sequencer (Applied Biosystems, Weiterstadt, Germany) [40].

Immunization of a 3-month-old female rabbit was performed with $4 \times 100\ \mu g$ purified and denatured antigen eluted from a preparative SDS gel (Elustar device, PHASE, Luebeck, Germany) [25]. Preparations from Boehringer Mannheim were used. First injections were made with complete Freunds adjuvant (days 1, 3, and 6) and booster injection on day 36 with incomplete adjuvant. High antibody titer antiserum was collected on days 50 and 54. Antibodies of the IgG class were prepared by use of Protein A Sepharose.

Antigen specific antibody preparation

Fractions containing immunoreactive protein against MLMR antibodies were separately loaded onto SDS gels (200 μg total protein per gel), electrophoresed and blotted as described. After blocking of unspecific binding sites, nitrocellulose sheets were incubated with antiMLMR antibody (1:1000) in TBST and washed 5×15 min. Dissociation of antibodies was achieved by washing the membrane with 100 mM glycine–HCl, pH 2.5 for 5 min. The resulting antigen specific antibody containing supernatant was removed and brought to a pH of 7.5 with 0.4 M Tris base.

Metyrapone reduction assay

Carbonyl reduction of metyrapone was performed in 50 μl volume containing the following: NADH 1 mM, NADPH 1 mM or 5 μl NADPH regenerating system [30 mg glucose-6-phosphate, 10 mg $NADP^+$, 0.5 ml 0.1 M $MgCl_2$, 475 μl 50 mM phosphate buffer, pH 7.4, 25 μl glucose-6-phosphate dehydrogenase (Boehringer Mannheim) per ml], metyrapone 1 mM, inhibitor 0.05 mM (dissolved in methanol) to give a final methanol concentration of 5%, 50 mM sodium phosphate buffer, pH 7.4 and 10–50 μg of protein. Samples were incubated for 20–30 min at 37°C, the reaction was stopped by addition of 150 μl icecold acetonitrile, mixed and centrifuged at 10,000 *g* for 10 min. 20 μl of the resulting supernatant was injected into a reversed phase HPLC system, consisting of a C18 (4.5×250 mm i.d.) column as stationary and 0.1% ammoniumacetate, pH 7.0, 30% acetonitrile as mobile isocratic phase. Peaks were detected with an UV/VIS monitor. The alcohol metabolite metyrapol elutes at 6.5 min and is clearly separated from the carbonyl substrate metyrapone, which elutes at about 10.0 min (flow rate 1.0 ml/min).

Limited proteolysis

Limited digestion of 15 μg purified MLMR was performed at 37°C for 10 h in 10% glycerol, 50 mM Tris glycine pH 6.8, 0.1% SDS with 30 ng staphylococcal V8 protease (Boehringer Mannheim). After digestion the sample was heated for 10 min to 100°C and subjected to a 17% PAGE system with tricine instead of glycine, giving a better resolution of small peptide fragments (cf. SDS–PAGE) [20].

Immunoblot analysis of separated peptides was carried out as described under Western blot, except that incubation of nitrocellulose sheets was performed in a PHASE screen blot apparatus (PHASE GmbH, Lübeck, Germany), which allows simultaneous screening of different antisera against a common antigen under identical conditions.

Protein determination

Protein determination was performed according to the method of Bradford [26] using BSA as standard.

RESULTS

Specific activities of carbony reduction and their relative inhibition by steroids

The ability to reduce carbonyl compounds was analyzed with the substrate metyrapone. Extracts were prepared from a number of different organisms. Microsomal fractions from liver of several vertebrate species and homogenates of larvae from an insect species were tested. The enzyme activities were compared to that of 3α-hydroxysteroid dehydrogenase purified from the bacterium *Pseudomonas testosteroni*. Alcohol metabolite formation was determined using an isocratic reversed phase HPLC system. Concentrations used in this study were 1.0 mM substrate, 50 μM inhibitor and different electron donating systems. In vertebrate liver microsomes a NADPH-regenerating system was used, except for guinea pig, where 1.0 mM NADPH was added. Specific activities (solvent control) and relative activities are given in Table 1. In vertebrate liver microsomes NADPH turned out to be the best cosubstrate whereas in the case of *Calliphora* homogenate and procaryontic HSD 28 1.0 mM NADH served as the

Table 1. Specific and relative activities of carbonyl reduction in different eucaryotic and procaryotic species and inhibition by different steroids

Source	Sp. act.	Range	Rel. act.	Residual activity (%)				
				5αD	5βD	5αP	5βP	ecd
Human	7.1	2.5–16.6	100	43	56	50	37	100
Pig	21.0	20.2–21.8	100	46	84	64	79	85
Sheep	19.6	19.2–20.0	100	69	87	79	73	116
Rabbit	16.6	15.1–17.5	100	50	61	57	50	95
Mouse	16.0	14.6–18.2	100	45	58	54	88	94
Rat	16.5	15.0–19.0	100	20	16	26	36	112
Guinea pig	32.8	28.9–33.6	100	68	54	72	47	100
Chicken	10.1	9.9–10.3	100	65	64	73	73	95
Blowfly	71.4	68.9–73.0	100	61	62	62	63	52
HSD 28	12,500	11,800–13,200	100	60	60	78	81	59

Carbonyl reduction of metyrapone was performed in 50 µl vol containing 1 mM substrate, 0.05 mM inhibitor (dissolved in methanol to give a final concentration of 5%) and various cosubstrates. In detail: in vertebrate liver microsomes a NADPH regenerating system was used (see Experimental), except for guinea pig, for which 1.0 mM NADPH was added. 1.0 mM NADH served as the electron supply in *Calliphora vicina* total homogenate and *Pseudomonas testosteroni* 3α-hydroxysteroid dehydrogenase preparations. Specific activity is expressed as nmol alcohol metabolite formed/30 min/mg protein. The uninhibited values were set at 100% and the relative inhibitions were expressed as residual activity in % of the uninhibited activity. Sources of fractions employed were vertebrate liver microsomes, *Calliphora vicina* (blowfly) larvae homogenate of day 6 and 3α-hydroxysteroid dehydrogenase (HSD 28) purified from *Pseudomonas testosteroni* (see Experimental). Values are mean values. Standard deviations amount to <7% in every case (except for human liver, see text). The number of independent samples (n) was as follows: mouse $n = 6$, rat $n = 6$, guinea pig $n = 6$, human $n = 6$, chicken $n = 2$, pig $n = 2$, sheep $n = 2$, rabbit $n = 4$, blowfly $n = 6$, HSD 28 $n = 6$. Abbreviations used: 5αD: 5α-dihydrotestosterone; 5βD: 5β-dihydrotestosterone; 5αP: 5α,3-oxo,20β-pregnanolone; 5βP: 5β,3-oxo,20β-pregnanolone; ecd: ecdysone.

electron supply. In the vertebrate fractions NADH could also serve as the cosubstrate, but specific activities were generally lower. Carbonyl reduction in *Calliphora* was strictly dependent upon NADH, whereas HSD 28 could also perform carbonyl reduction with NADPH, although to a much lower extent (about 1/10 of specific activity) (data not shown).

Alcohol metabolite formation was linear at least over a time course of 30 min of incubation (data not shown).

The specific activities in vertebrate liver microsomes range from 7.1 in humans up to 32.8 nmol/30 min/mg protein in guinea pigs. Higher specific activities are obtained in the total homogenate of *Calliphora vicina* day 6 larvae (71.4 nmol/30 min/mg) and with purified 3α-hydroxysteroid dehydrogenase from *Pseudomonas testosteroni* (12.5 µmol/30 min/mg).

Steroids of the androstane or pregnane class with A/B ring *cis* or *trans* conformation as well as the insect steroid hormone ecdysone served as inhibitors. In vertebrate liver microsomes both classes of vertebrate-type hormones inhibited carbonyl reduction to different degrees (cf. Table 1). Ecdysone had only slight effects in these fractions except for rat, pig and sheep. The inhibition (pig) and increase in activity (sheep) might by explained by the low sample number ($n = 2$), but no explanation can be given for the significant increase in activity in rat liver microsomes ($n = 6$). Maybe the ecdysteroid solubilizes the enzyme in the membrane thus giving better access to the active site. The insect homogenate and HSD 28 from *Pseudomonas testosteroni* could be inhibited by all the steroids employed in this study.

Immunoblot with antiMLMR antibodies

Microsomal and cytosolic liver fractions of the vertebrate species tested as well as that of *Calliphora* homogenate, *Calliphora* brain ring gland complex and a prepurified extract of *Pseudomonas spec.* were separated on SDS–PAGE and transferred to a nitrocellulose membrane. The blot was probed with an affinity purified antibody against MLMR. Figure 1 shows the result of this immunoblot. No immunological crossreactions were observed in the vertebrate liver cytosols, but in the microsomal liver fractions of all vertebrate species a specific antigenic recognition occurred in the range of 30–35 kDa. In the prepurified *Pseudomonas* fraction a strong antibody binding was observed with a 28 kDa protein. In *Calliphora* total homogenate or brain ring gland complex specific crossreactions were observed with a protein band migrating in the 34 kDa region.

Antigen specific antibody purification and limited proteolysis of MLMR

To search for conserved domains within the MLMR protein, which probably represent the antigenic determinants and which are supposed to be common in all tested fractions (cf. Fig. 1) antigen specific antibody purification was

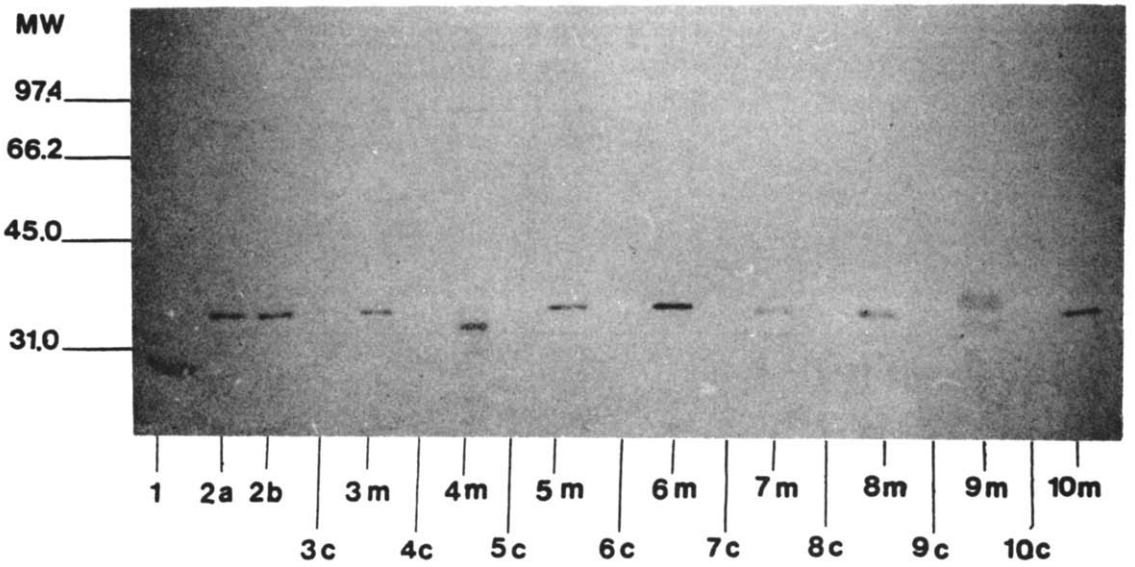

Fig. 1. Immunoblot of eucaryotic and procaryotic fractions probed with antiMLMR antibodies. 25 μg total protein per lane were separated on a 10% SDS–PAGE and blotted to nitrocellulose as described in Experimental. Lane 1: prepurified extract of *Pseudomonas testosteroni*; 2: *Calliphora vicina*, a = total larvae homogenate (late third instar), b = brain ring gland complexes (late third instar); 3: chicken liver; 4: guinea pig liver; 5: rat liver; 6: mouse liver; 7: pig liver; 8: sheep liver; 9: rabbit liver, 10: human liver. c = cytosol, m = microsomes.

performed. Figure 2 gives the result of an immunoblot with the different affinity purified antibodies, all probed against mouse liver microsomal proteins (for numbering of the lanes see legend). Each of the antibodies showed a specific crossreaction in the 34 kDa region

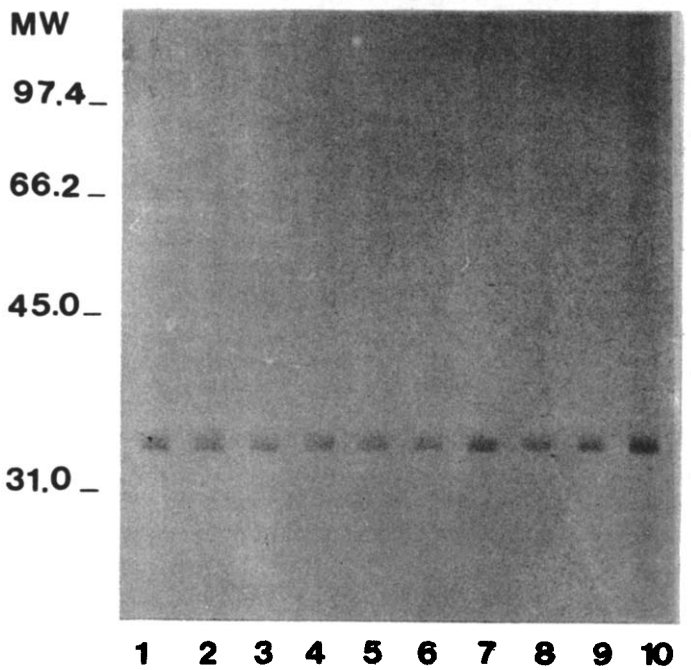

Fig. 2. Immunoblot with affinity purified antibodies. 100 μg microsomal protein of mouse liver were separated by SDS–PAGE (the gel was poured without sample comb so the protein sample could be applied across the entire width of the gel) and blotted to nitrocellulose. The antibodies were derived from affinity purification using crossreacting proteins of the respective fractions (shown in Fig. 1) as specific antigen. (For details see Experimental.) A screenblot apparatus from PHASE (Luebeck, Germany) was used for simultaneous and convenient screening of the different antibodies against the same antigen. Numbering of affinity purified antibodies as follows: Lane 1: antimouse; 2: antirat; 3: antiguinea pig; 4: antisheep; 5: antichicken; 6: antipig; 7: antirabbit; 8: anti*calliphora*; 9: antiHSD 28; 10: antihuman.

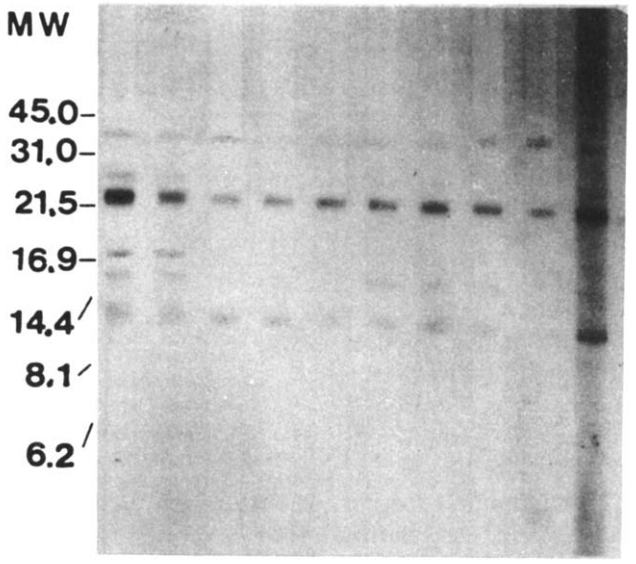

Fig. 3. Immunoblot of partially digested MLMR protein probed with different affinity purified antibodies. 15 μg purified MLMR were partially digested with staphylococcal V8 protease. The resulting fragments were separated on a 17% SDS tricine based polyacrylamide gel and immobilized on nitrocellulose as described in Experimental. Molecular weight markers employed were from Pharmacia LKB (peptide markers) or from Bio Rad (protein low standard). Numbering of lanes corresponds to Fig. 2.

corresponding to the molecular mass of the MLMR.

In Fig. 3 the same antibodies were probed against a Western blot of MLMR fragments after limited digest with staphylococcal V8 protease and separation on a 17% polyacrylamide gel (see Experimental). Lanes 1 and 2 show that 6 fragments derived from the proteolytic MLMR digest were recognized by the mouse and rat antigen specific antibodies, respectively. One 22 kDa fragment crossreacted with the affinity purified antibodies of all species, as it did the non-digested MLMR in the 34 kDa region. The degree of recognition of other specific antibodies varied depending upon the species.

Immunoblot with antibodies against 3α-hydroxysteroid dehydrogenase from Pseudomonas testosteroni

Vertebrate liver microsomes (chicken liver was omitted) as well as *Calliphora* and *Pseudomonas* fractions were analyzed by the immunoblot technique using antiHSD 28 IgG antibodies (see Fig. 4). Essentially the same

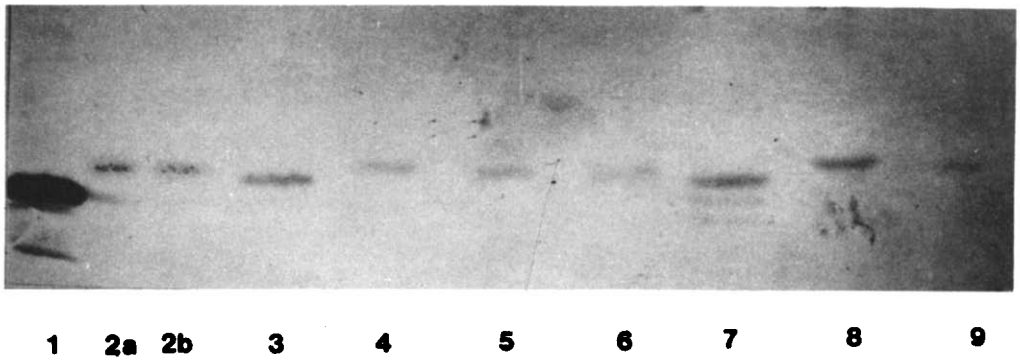

Fig. 4. Immunoblot of vertebrate liver microsomes, *Calliphora* and *Pseudomonas testosteroni* fractions, probed with an antiHSD 28 IgG antibody. 25 μg protein per lane was separated on 10% SDS–PAGE and blotted to nitrocellulose. Numbering as follows: Lane 1: prepurified HSD 28 fraction; 2: *Calliphora vicina*, a = total homogenate, b = homogenate of brain ring gland complex; 3: guinea pig; 4: rat; 5: mouse; 6: pig; 7: sheep; 8: rabbit; 9: human (chicken was omitted in this experiment).

crossreaction pattern was observed when compared to Fig. 1, suggesting that the antiHSD 28 IgG antibodies possess similar antigenic specificities as the antiMLMR antibodies.

Carbonyl reduction, inhibition by 3β-hydroxy-desogestrel and expression of metyrapone reductase in human liver microsomes

To determine the role of human liver microsomal metyrapone reductase in the metabolism of the contraceptive steroid desogestrel Lineweaver–Burk kinetic analysis was performed with 6 different human liver microsomal samples, derived from both sexes and varying ages of the source (for details see legend of Fig. 5). The respective uninhibited specific activities were set at 100% and the relative inhibition data were calculated. This kind of calculation was necessary because the absolute specific activities exhibit a wide range of variation. 3α- and 3β-hydroxy-desogestrel were used as inhibitors. The 3α isomer exhibited almost no effect (highest inhibition obtained was 15% compared to control) on carbonyl reduction of metyrapone, whereas the 3β-hydroxy-desogestrel showed a competitive kind of inhibition. The graph of Fig. 5 shows the competitive inhibition of carbonyl reduction by 3β-hydroxy-desogestrel with a K_m of 0.59 mM and a K_i of 20 μM.

In two of the human liver microsomal fractions, obtained from two neonatal male probes

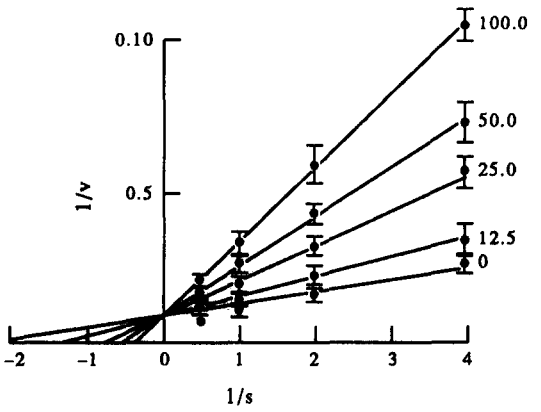

Fig. 5. Lineweaver–Burk plot of carbonyl reduction of metyrapone in human liver microsomes and inhibition by 3β-hydroxy-desogestrel. 6 different human liver samples were used (number 1, 2, 3, 4, 7, 8 as shown in Fig. 6). Specific activities were set at 100% (substrate concentration 2 mM) and relative values in the absence and presence of inhibitor were determined. Inhibitor concentrations used were 12.5, 25.0, 50.0 and 100.0 μM 3β-hydroxy-desogestrel. Individual specific activities are listed below in the legend to Fig. 6.

(numbers 5 and 6), neither carbonyl reduction of metyrapone nor immunoreactivity against antiMLMR antibodies could be observed. In Fig. 6 the expression of the metyrapone reductase in human liver microsomes was detected in the 34 kDa region of the immunoblot. The intensity of crossreacting protein bands, probed with the antibody against the microsomal MLMR, always paralleled the specific activities of metyrapone reduction within these fractions.

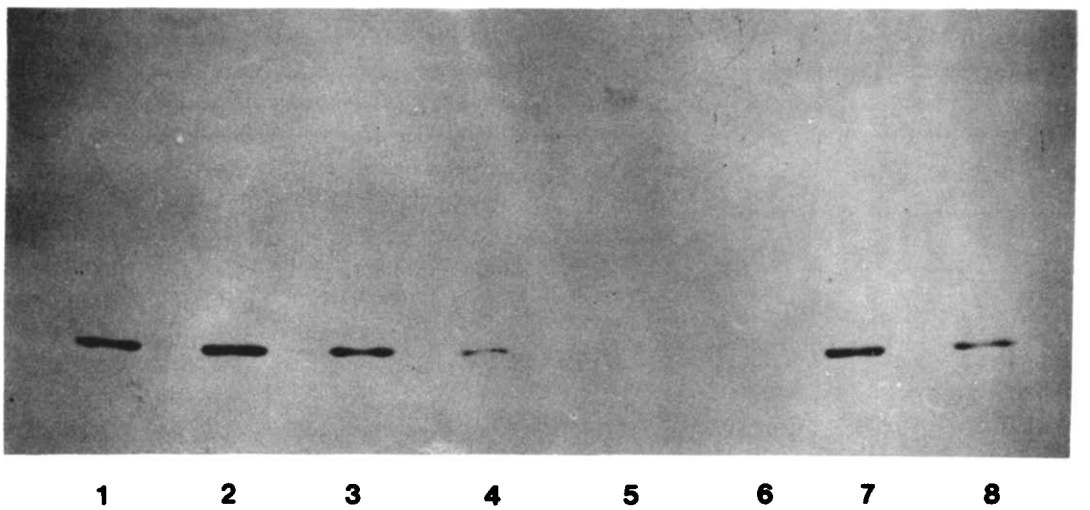

Fig. 6. Western blots of 8 individual human liver microsomal samples with antiMLMR antibodies: 25 μg of microsomal protein per lane was separated on 10% SDS–PAGE and immobilized on nitrocellulose. Antibody incubation was with antiMLMR antibody. Numbering and characterization of the samples as follows: Lane 1: f, 36 y, 6.95; lane 2: f, 61 y, 16.6; lane 3: f, 90 y, 5.2; lane 4: f, 91 y, 2.6; lane 5: m, 39 w; n.a.; lane 6: m, 0.5 y, n.a.; lane 7: m, 70 y, 9, 2; lane 8: m, 86 y, 2.5. (abbreviations: f = female, m = male; w = age in weeks, y = age in years; n.a. = no activity detected, or specific activity in nmol/30 min/mg protein).

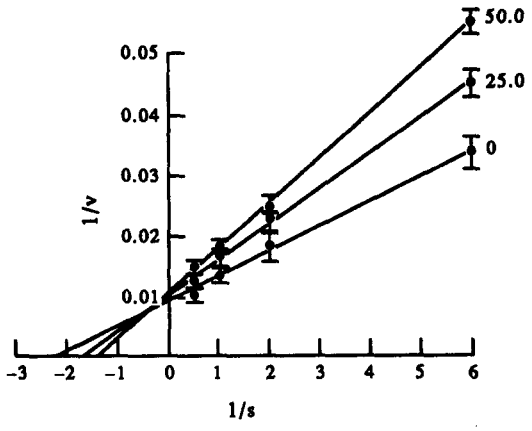

Fig. 7. Lineweaver–Burk plot of carbonyl reduction in *Calliphora vicina* larvae homogenate. Homogenate was incubated with different concentrations of the substrate metyrapone in the absence and presence of varying concentrations of ecdysone. Data were converted to their reciprokes and plotted by use of the Graph Pad PC software. A similar kind of inhibition was observed when the ecdysone inhibition of HSD 28 carbonyl reduction was investigated. (Data not shown.)

Inhibition of carbonyl reduction by ecdysone in larvae homogenate of Calliphora vicina

Figure 7 shows the kinetics of carbonyl reduction by homogenate of blowfly larvae in the presence of ecdysone. Ecdysone showed the properties of a mixed-type inhibitor. The same kind of inhibition was achieved when 3α-hydroxysteroid dehydrogenase from *Pseudomonas testosteroni* was employed.

DISCUSSION

In the past decade several attempts have been made for the characterization of liver cytosolic carbonyl reductases including the determination of primary structures of several proteins [11, 27 and references therein]. Those resulted in the establishment of the aldo-keto reductase superfamily which is involved in the metabolism of endogenous and exogenous carbonyl and quinone compounds. Homologies to this family were found when the primary structure of the indomethacine sensitive cytosolic rat liver 3α-hydroxysteroid dehydrogenase/*trans* dihydrodiol dehydrogenase was elucidated [3–5]. This enzyme has properties concerning the catalytic conversion of 3α-hydroxysteroids, prostaglandins, enzymatic inactivation of ultimate carcinogenic polyaromatic hydrocarbons and bile acid binding [4, 6]. Up to now little is known about mammalian microsomal carbonyl reduction, although several proteins involved in this process were purified and described [7–10]. Sawada's group [7–9] demonstrated the existence of several different carbonyl reductases in the microsomal fractions of guinea pig and rat liver which also accepted steroids with high affinity as substrates and which were classified as 3α-hydroxysteroid dehydrogenases.

In previous investigations we established the existence of an immunologically related enzyme species in liver microsomes of both rodents and humans [12, 13], capable of carbonyl reduction and susceptible to inhibition by steroids of the androstane class which shows the same features as already described in guinea pig and rat liver by Sawada's group [7–9]. Immunoblot experiments with an antibody against the microsomal MLMR as well as functional assays revealed that a 3α-hydroxysteroid dehydrogenase from *Pseudomonas testosteroni* also shares structural and functional properties with this class of microsomal carbonyl reductases [17].

The intention of this study was to evaluate and describe the functional and structural features of this class of enzymes in different phylogenetic species. For this purpose we determined the extent of carbonyl reduction and its inhibition by several steroids in liver microsomes of several vertebrate species as well as in insects and procaryonts. It was found that carbonyl reduction could be strongly inhibited (i.e. at least to 40–65%) not only by androstanes but also by steroids of the pregnane class. This was observed in every tested fraction even when a 20-fold excess of substrate over inhibitor was used. Ecdysteroids, e.g. ecdysone, had no inhibitory effect on carbonyl reduction in vertebrate liver microsomes, but inhibited this reaction significantly in the case of the insect and bacterial enzyme, where it showed a mixed type of inhibition. The immunoblot experiments revealed common antigenically related proteins in vertebrate liver microsomes and, surprisingly, in *Calliphora vicina* and *Pseudomonas testosteroni*, when probed with the antibody directed against MLMR. A similar pattern of crossreactivity is observed when the antibodies against purified 3α-hydroxysteroid dehydrogenase from *Pseudomonas* were used. The mutual immunoreactivities of the two antibody species against the tested proteins suggest the existence of structural homologies within these carbonyl reducing enzymes derived from vertebrate liver, insects and procaryonts. A conserved domain of the vertebrate protein might be located within a 22 kDa fragment generated with V8 protease. This fragment is recognized specifically by each

affinity purified antibody preparation. These facts together with the strong inhibition by steroids, further point to a related protein family which is located in the endoplasmatic reticulum of vertebrate liver and which is engaged in the metabolism of endogenous and/or exogenous carbonyl compounds. Moreover, based on the obvious functional and structural homologies to procaryontic or lower eucaryontic proteins, these enzymes might be derived from a common ancestor.

The proteins characterized might belong to those hydroxysteroid dehydrogenases, which are already described in the species investigated in this study [24,28–34]. Up to now no hydroxysteroid dehydrogenase activity of the purified MLMR could be demonstrated [17]. But its strong inhibition by steroids [17], however, and the identity of metyrapone reductase and 3α-hydroxysteroid dehydrogenase in rat and guinea pig [7–9] along with structural properties discovered in immunoblot with antiMLMR antibodies [12], as well as the structural and functional homologies to HSD 28 from *Pseudomonas testosteroni* make it certain that these proteins belong to the same class. The competitive inhibition of metyrapone reduction by 3β-hydroxy-desogestrel suggests that the metyrapone reductase in human liver microsomes participates in the bioactivation of this widely employed contraceptive steroid [34]. Further purification of the respective enzyme(s) from human sources should reveal the exact structural requirements for the physiological substrates.

The strong inhibition of metyrapone reduction in *Calliphora* by ecdysteroids—although being of a mixed type—also suggests a role in ecdysteroid metabolism, which might be—besides as inhibitor of 20-hydroxy monooxygenase—a participation in the ecdysone epimerase complex, namely as 3-dehydroecdysone reductase which converts ecdysone via dehydroecdysone to 3α-ecdysteroids thus inactivating the moulting hormone [30, 31]. This point is of considerable interest, because insecticide substances based upon the metyrapone structure are under current development [35], and further experiments with metyrapone or its analogues in insect bioassays will determine the significance of such compounds in plant protection.

The expression and detection of MLMR in microsomal fractions of cutaneous tissue in mouse and human samples as revealed by Western blot analysis and immunocytochemistry, point to a role of this enzyme in steroid and/or xenobiotic metabolism also in other organs [36].

Further interest is attracted on the enzyme family of "short chain" or "insect type" alcohol dehydrogenases. Up to now several bacterial as well as some lower and higher eucaryontic dehydrogenases were shown to belong to this group, for example 3β-hydroxysteroid dehydrogenase from *Pseudomonas testosteroni*, *cis*-benzene dihydrodiol dehydrogenase from *Pseudomonas pseudoalcaligenes*, 17β and 11β-hydroxysteroid dehydrogenase from human tissues [37–39]. Interestingly the N-terminal amino acid sequence of HSD 28 shows striking homologies to a member of this family. Preliminary sequence data of MLMR also reveal homologies to another member of this protein class, so it might be possible that the proteins described in this paper constitute a subgroup of the short chain alcohol dehydrogenase family. Nevertheless, further sequence data will reveal their exact place in existing protein families. The evaluation of the primary structure of MLMR and HSD 28 as well as their exact stereo and substrate specificities are under current research in our laboratory.

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