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Functional and immunological relationships between metyrapone reductase from mouse liver microsomes and 3α-hydroxysteroid dehydrogenase from *Pseudomonas testosteroni*[1]

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3α-Hydroxysteroid dehydrogenase (3α-HSD) from *Pseudomonas testosteroni* was shown to reduce the xenobiotic carbonyl compound metyrapone (MPON). Reversely, MPON reductase purified from mouse liver microsomes and previously characterized as aldehyde reductase, was competitively inhibited by 3α-HSD steroid substrates. For MPON reduction both enzymes can use either NADH or NADPH as co-substrate. Immunoblot analysis after native and SDS gel electrophoresis of 3α-HSD gave a specific crossreaction with the antibodies against the microsomal mouse liver MPON reductase pointing to structural homologies between these enzymes. In conclusion, there seem to exist structural as well as functional relationships between a mammalian liver aldehyde reductase and prokaryotic 3α-HSD. Moreover, based on the molecular weights and the co-substrate specificities microsomal mouse liver MPON reductase and *Pseudomonas* 3α-HSD seem to be members of the short-chain alcohol dehydrogenase family.

Carbonyl reduction; Steroid metabolism; Aldehyde reductase; 3α-Hydroxysteroid dehydrogenase; *Pseudomonas testosteroni*; Metyrapone

1. INTRODUCTION

Carbonyl reducing enzymes constitute the enzyme family of the aldo-keto reductases, which catalyze the reduction of carbonyl group bearing substrates to the corresponding alcohols. They are distributed in various mammalian and non-mammalian tissues and share typical features such as monomeric structure in the molecular weight region between 30–40 kDa, almost exclusive dependence on NADPH as co-substrate and cytosolic subcellular localization [3,4]. They have been originally referred to as carbonyl reductase and metabolize a broad spectrum of exogenous carbonyl compounds, including aliphatic and aromatic aldehydes and ketones, where they obviously play an important role in detoxification processes. Furthermore, enzymes like NAD(P)H: quinone-oxidoreductase (NQR, EC 1.6.99.2), dihydrodiol dehydrogenase (DDH, EC 1.3.1.20), and hydroxysteroid dehydrogenases might be involved in carbonyl metabolism. In addition to their contribution to the detoxification process carbonyl reductases participate in the reductive metabolism of a great variety of physiologically occurring endogenous substrates and were shown to catalyze the carbonyl reduction of prostaglandins [5,6], biogenic amines [7] and steroid hormones [8–10]. However, their physiological role is not fully understood and remains to be determined.

We use the ketone metyrapone (MPON), the potent cytochrome P-450 inhibitor, as a substrate to investigate carbonyl reducing enzymes. In previous studies we purified and characterized a MPON reducing enzyme from mouse liver microsomes and demonstrated that this enzyme belongs to the aldo-keto reductase family [11]. Differing from common characteristics of this enzyme family the MPON reductase from mouse liver microsomes has the ability of utilizing both NADPH and/or NADH as co-substrates and is localized in the endoplasmic reticulum of the cell. Moreover, although reducing a ketone, the enzyme was characterized as an aldehyde reductase due to its sensitivity to the diagnostic inhibitor phenobarbital [4] and according to the inhibitor classification of the aldo-keto reductases.

In this study the possible physiological function of the

[1]Although reclassified as *'Comamonas testosteroni'* [1], the original name *'Pseudomonas testosteroni'*, as described and grouped by Marcus and Talalay [2], is used in this paper.

Abbreviations: 3α-HSD, 3α-hydroxysteroid dehydrogenase; HPLC, high-performance liquid chromatography; SDS, sodium dodecyl sulfate; androsterone, 5α-androstan-3α-ol-17-one; androstandione, 5α-androstan-3,17-dione; 5α-DHT, 5α-dihydrotestosterone; 5β-DHT, 5β-dihydrotestosterone; MPON, metyrapone; MPOL, reduced metyrapone (metyrapol).

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liver microsomal MPON reductase was of interest, in order to promote the understanding of the physiological role of the aldo-keto reductases. The potentiality of dual co-substrate utilization and the ability of 5α-dihydrotestosterone to competitively inhibit MPON reduction suggest this enzyme to be related to 3α-hydroxysteroid dehydrogenase (3α-HSD) [11]. Therefore, to investigate functional similarities between these two enzymes, we tested 3α-HSD from *Pseudomonas testosteroni* for its ability to reduce MPON. Reversely, MPON reductase from mouse liver microsomes was tested for its sensitivity to steroid substrates of 3α-HSD, such as testosterone, 5α-dihydrotestosterone, 5β-dihydrotestosterone, progesterone, androstandione and androsterone, respectively for its ability to catalyze the oxidoreduction of these compounds. Furthermore, in order to obtain information about immunological and thus structural homologies between 3α-HSD and MPON reductase, we checked antibodies against the microsomal mouse liver enzyme for crossreactivity with the *Pseudomonas* protein.

2. MATERIALS AND METHODS

2.1. *Animals*

Livers of female NMRI mice were used throughout the study. 3α-hydroxysteroid dehydrogenase (3α-HSD) from *Pseudomonas testosteroni* was purchased from Boehringer Mannheim (Mannheim, Germany) (Lot N°. 10691627-01) or Sigma (Deisenhofen, Germany) (Grade II; N°. 79c-6820).

2.2. *Chemicals*

NADH, NADPH, G-6-P and G-6-P-DH were obtained from Boehringer Mannheim (Mannheim, Germany). For HPLC acetonitrile of HPLC-grade from E. Merck (Darmstadt, Germany) was used. Low molecular weight markers were obtained from BioRad (Munich, Germany), SDS from Fluka (Buchs, Switzerland), acrylamide, methylenebisacrylamide, 4-chloro-1-naphthol and H_2O_2 from E. Merck (Darmstadt, Germany), nitrocellulose from Schleicher and Schuell (Dassel, Germany), testosterone, androsterone, androstandione, progesterone, 5α-dihydrotestosterone. 5β-dihydrotestosterone, Meldola blue (8-dimethyl-2,3 benzophenoxazin), *p*-iodotetranitrozolium from Sigma (Deisenhofen, Germany), and peroxidase-conjugated anti-rabbit IgG antibodies from Dakopatts (Hamburg, Germany). All other chemicals used in the experiments were reagent grade and were obtained by commercial suppliers.

2.3. *Purification of the microsomal MPON reductase*

Purification of the microsomal MPON reductase for enzymatic assays and for immunization was performed as described elsewhere [11,12].

2.4. *Immunization and preparation of antisera*

1 mg of the enzyme preparation was dissolved in 62.5 mM Tris-HCl, 0.1% (w/v) SDS, 0.5 mM EDTA, pH 6.8 and emulsified with 1 Vol. complete Freund's adjuvant. For immunization, a female rabbit was injected subcutaneously (0.9 ml emulsion containing about 0.3 mg protein (2 ×) or 0.2 mg protein (2 ×) per injection) four times on the following days: 1, 3, 5 and 36, the latter being the booster.

2.5. *SDS-polyacrylamide gel electrophoresis (SDS-PAGE), electroelution and immunoblotting*

SDS-PAGE [13], electroelution of proteins [14] and immunoblotting [15] were performed as described previously [12].

2.6. *Native gel electrophoresis*

Native gel electrophoresis was carried out as described under SDS-PAGE, but SDS was omitted. Gels were run at 4°C with 185 V constant voltage.

2.7. *Activity staining after native gel electrophoresis*

(A) 3α-HSD activity was detected by incubating the gel slices in a solution containing 1.5 mM NAD^+, 0.5 mM androsterone (dissolved in methanol), 0.05 mM Meldola blue (8-dimethyl-2,3 benzophenoxazin) and 0.31 mM *p*-iodonitrotetrazolium. Enzymatically active protein bands are seen as purple formazan bands.

(B) MPON reducing activity was detected by preincubating the gel slices for 30 min in a solution containing 1.5 mM NADH, 1.5 mM NADPH and 2.3 mM MPON. Visualization of MPON reducing enzymes was performed by incubation in a solution containing 0.05 mM Meldola blue, 10 mM sodium phosphate buffer, pH 7.4, and 0.31 mM *p*-iodonitrotetrazolium. Enzymatically active protein bands are seen as transparent bands against a purple background.

2.8. *Immunoblot after native gel electrophoresis*

Gel slices were incubated for 30–60 min in a solution containing 375 mM Tris/HCl, pH 8.8, and 0.5% SDS and blotted for 2 h in a semidry apparatus according to Kyse-Anderson [15].

2.9. *Enzyme assays*

Assay of MPON reduction and subsequent reduced metabolite detection by HPLC was performed as described previously [12].

2.10. *Protein determination*

Protein concentration was determined by the method of Lowry et al. [16].

3. RESULTS

3.1. *MPON reduction by 3α-HSD*

As can be seen in Table I the *Pseudomonas* 3α-HSD preparation has the ability of reducing the xenobiotic carbonyl compound MPON to the respective alcohol metabolite metyrapol (MPOL). Compared to the mouse liver enzyme V_{max} values are almost similar, but K_m values differ considerably: whereas MPOL formation by mouse liver MPON reductase has a much lower K_m with NADPH than NADH as co-substrate, that of 3α-HSD is slightly lower with NADH. This corresponds to other reports, which demonstrate that 3α-HSD of *Pseudomonas testosteroni* in principle is dependent on

Table I

Reduction of MPON by 3α-hydroxysteroid dehydrogenase (3α-HSD) from *Pseudomonas testosteroni* and MPON reductase from mouse liver microsomes.

Enzyme	Co-substrate	K_m (mM)	V_{max} (nmol/mg/30 min)
MPON reductase	NADH	4.77	347.83
	NADPH	0.22	211.35
3α-HSD	NADH	0.56	352.65
	NADPH	0.64	314.60

Enzyme activities were assayed in the standard reaction mixture containing 50 mM sodium phosphate buffer, pH 7.4, MPON concentrations between 0.05–3.2 mM and either 3.2 mM NADH or 3.2 mM NADPH, respectively. Reduced metabolite detection was performed by HPLC analysis.

Table II

Inhibition of MPON reduction by steroid substrates of 3α-HSD.

Steroid substrate	Residual activity (%)
Testosterone	23.2
Androstandione	30.4
Progesterone	35.0
5β-Dihydrotestosterone	39.4
5α-Dihydrotestosterone	47.3
Androsterone	56.6

Enzyme activities were assayed in the standard reaction mixture containing 50 mM sodium phosphate buffer, pH 7.4, 0.5 mM MPON, a NADPH-regenerating system and 0.5 mM of the respective inhibitor. The percentages were calculated from uninhibited control experiments. Control velocities were determined in the presence of appropriate quantities of the solvents. Reduced metabolite detection was performed by HPLC analysis.

NADH rather than on NADPH as electron donor [17–19].

3.2. *Inhibition of MPON reductase by steroids*

As shown in Table II, purified MPON reductase from mouse liver microsomes is strongly inhibited by steroid substrates of 3α-HSD, such as androsterone, testosterone, 5β-DHT, 5α-DHT, progesterone and androstandione. The competitive nature of this inhibition (determined for the latter three compounds) gives rise to the suggestion that steroids are physiological substrates of this enzyme. But when tested, neither of these substances was metabolized by microsomal MPON reductase, as indicated by TLC, HPLC or indirect spectrophotometric recording of pyridine nucleotide oxidoreduction.

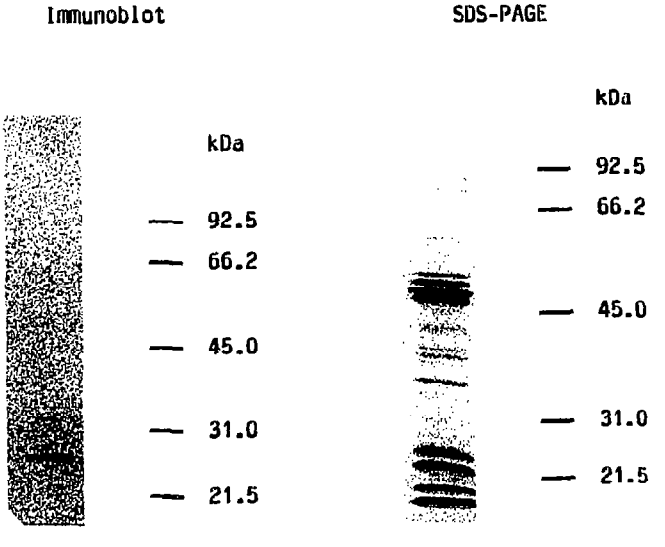

Fig. 1. SDS-PAGE and immunoblot of 3α-hydroxysteroid dehydrogenase (3α-HSD) from *Pseudomonas testosteroni*. The antibody against the microsomal mouse liver metyrapone reductase crossreacted specifically with the 3α-HSD in the 28 kDa molecular weight region. For details see section 2.

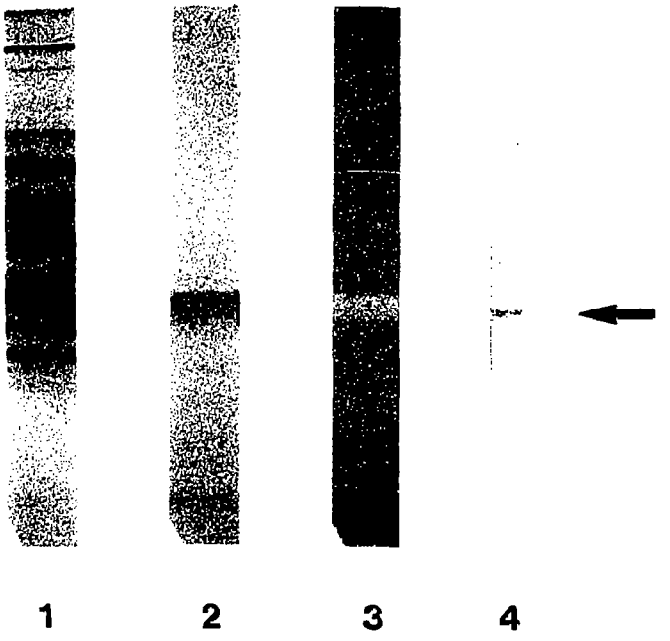

Fig. 2. Activity staining of 3α-hydroxysteroid dehydrogenase (3α-HSD) from *Pseudomonas testosteroni* after native gel electrophoresis. Lane 1, Coomassie stained protein pattern of the 3α-HSD preparation; lane 2, androsterone oxidating activity of 3α-HSD; lane 3, metyrapone reducing activity of 3α-HSD; lane 4, immunoblot of 3α-HSD after native gel electrophoresis using the antibody against the microsomal mouse liver metyrapone reductase. For details see section 2.

3.3. *Immunochemical analysis of 3α-HSD*

Fig. 1 shows that the polyclonal antibodies against the microsomal mouse liver reductase also crossreacted specifically with the *Pseudomonas* 3α-HSD in the 28 kDa molecular weight region [17], indicating the presence of common antigenic determinants. Fig. 1 also reveals that the commercially available 3α-HSD preparation of *Pseudomonas testosteroni* still consists of a complex pattern of protein bands.

3.4. *Native gel electrophoresis and activity staining of 3α-HSD*

To demonstrate that the 28 kDa band is associated with the 3α-HSD activity and that the same band also represents the MPON reducing activity native gel electrophoresis (NGE) and subsequent activity staining were performed. Fig. 2 shows in the first lane the Coomassie stained protein pattern of 3α-HSD preparation of *Pseudomonas testosteroni* after NGE. The steroid dehydrogenase activity in the gel slice was detected using androsterone as substrate [17] which is oxidized at the 3α-OH group of Ring A of the steroid molecule and which results in a group of three purple bands of lane 2. Lane 3 shows a gel slice of the same enzyme preparation, but was incubated with MPON as

substrate, which is reduced at its ketone function to the corresponding alcohol metabolite MPOL. Activity staining revealed a broad transparent band against a purple background in the same region, representing the carbonyl reducing activity of the dehydrogenase. The two corresponding enzyme activities within the same protein band indicate that 3α-HSD from *Pseudomonas testosteroni* is capable of oxidizing androsterone as well as reducing the ketone MPON.

3.5. *Immunoblotting after NGE*

Immunoblotting after NGE of the *Pseudomonas* dehydrogenase with the antibody against the mouse liver enzyme gave a positive crossreaction in the same region (Fig. 2) verifying the suggestion that there exist structural homologies between the MPON reductase from mouse liver microsomes and the 3α-HSD from *Pseudomonas testosteroni*, which indicates a possible relationship between these enzymes.

The identity of this enzyme with 3α-HSD was proved again by cutting out the respective protein band of the native gel and subjecting it to electroelution followed by SDS-PAGE and subsequent immunological characterization. Corresponding to the results of Fig. 1 SDS-PAGE revealed a protein band in the 28 kDa molecular weight region, which was immunoreactive to the antibodies against the mouse liver enzyme (data not shown).

In some immunoblot experiments after NGE of 3α-HSD an additional band of crossreaction was observed in the higher molecular weight region, but no band of activity, neither with androsterone nor with MPON as a substrate, could be observed in this region. This corresponds to Skålhegg [20] who provided evidence that 3α-HSD of *Pseudomonas testosteroni* exhibits reversible concentration-dependent monomer–dimer transitions, which resulted in a 90% reduction of specific activity in the case with androsterone as substrate. After lowering the protein concentration in the solution for NGE the higher molecular weight band after immunoblotting could not be detected in our experiments, probably due to the dissociation of the dimers into the active monomers.

As described already by Shikita and Talalay [17], the 3α-HSD of *Pseudomonas testosteroni* resides in two major and one minor catalytic activities after NGE, which corresponds to the present results with androsterone as substrate for oxidation (Fig. 2). However, this hint to the existence of various isozymes could not be supported with MPON as substrate for carbonyl reduction, which might be due to methodological problems (diffusion of pyridine nucleotides during the preincubation period; cf. section 2).

4. DISCUSSION

The present studies have demonstrated that 3α-HSD from *Pseudomonas testosteroni* and MPON reductase from mouse liver microsomes on the one hand share similar functional properties in that both are able to catalyze MPON reduction, respectively that steroid substrates of 3α-HSD proved to be strong and competitive inhibitors of the mammalian liver enzyme. In addition, both show dual co-substrate specificity for NADH or NADPH. On the other hand, MPON reductase and 3α-HSD seem to be structurally related due to the antigenic crossreaction in immunoblot experiments.

The characterization of aldo-keto reductases often revealed that former classifications based on functional data do not always correspond to phylogenetic classifications based on structural data. In contrast, despite lacking enzyme properties some proteins could be classified as members of respective oxidoreductase families.

In general, the following superfamilies can be distinguished: (1) aldo-keto reductases comprising human liver aldehyde reductase, human placenta aldose reductase, rat lens aldose reductase [21,22], 2,5-diketogluconic acid reductase [23], prostaglandin F synthetase [24], and frog *p*-crystallin [25]; (2) short-chain alcohol dehydrogenases comprising 17β-hydroxysteroid dehydrogenase, 15-hydroxyprostaglandin dehydrogenase, Nod G protein of *Rhizobium meliloti* [26] and 3β-hydroxysteroid dehydrogenase from *Pseudomonas testosteroni* [27]; (3) NAD(P):quinone oxidoreductase, hitherto the sole member of this family.

For a considerable number of carbonyl reducing enzymes the classification to one of these families is unknown. We propose a relationship of the enzymes investigated in this study to the family of short chain alcohol dehydrogenases, based on the low molecular weights (28 kDa for 3α-HSD from *Pseudomonas testosteroni* and 34 kDa for MPON reductase from mouse liver microsomes) and their co-substrate specificities.

In summary, besides exhibiting structural as well as functional relationships microsomal mouse liver MPON reductase and *Pseudomonas* 3α-HSD possibly belong to the short-chain alcohol dehydrogenase superfamily.

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