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CARBONYL REDUCTION OF METYRAPONE IN HUMAN LIVER

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Abstract—Carbonyl reduction was investigated in cytosolic and microsomal fractions of human liver using the ketone metyrapone as a substrate. The cytosolic enzyme has a stronger preference for NADPH over NADH than the microsomal enzyme: the former shows only 14% of the NADPH-supported activity while the latter exhibits 36% activity with NADH. Barbitone and quercitrin, the classic inhibitors of carbonyl reductases, do not affect metyrapone reduction in either fraction. Dicumarol and indomethacin, the specific inhibitors of NAD(P)H:quinone-oxidoreductase and dihydrodiol dehydrogenase, respectively, only slightly decreased metyrapol formation. In contrast, 5α-dihydrotestosterone, the active form of the androgen steroid testosterone, inhibited metyrapone reduction very strongly in the microsomal fractions and is postulated to be the physiological substrate of the enzyme. This resembles the situation in mouse liver [E. Maser and K. J. Netter, *Biochem Pharmacol* **38**: 3049–3054, 1989] where microsomal metyrapone reductase was inhibited by steroids and the purified enzyme was demonstrated to mediate androsterone oxidation. Immunoblot analysis revealed antigenic cross-reaction of antibodies against the 34 kDa metyrapone reductase from mouse liver microsomes with the homologous protein in human liver microsomes pointing to structural homologies between the respective enzymes of the two species. These results—together with previous findings, which have shown that there exist functional as well as structural relationships between microsomal mouse liver metyrapone reductase and 3α-hydroxysteroid dehydrogenase from *Pseudomonas testosteroni* (E. Maser, U. Oppermann and K. J. Netter, *Eur J Pharmacol* **183**: 1366, 1990]—suggest that metyrapone reduction in human liver microsomes might be catalysed by a microsomal hydroxysteroid dehydrogenase.

Metabolic reduction is an important step in the biotransformation of carbonyl group bearing substrates in many mammalian and non-mammalian tissues [1, 2]. Xenobiotic aromatic and aliphatic aldehydes and ketones are often reduced to the corresponding alcohols prior to their further metabolism and elimination. In addition, endogenous substances such as prostaglandins [3], biogenic amines [4] and steroids [5, 6] were shown to undergo this step of metabolic reduction. The enzymes involved in carbonyl reduction are summarized in the family of carbonyl reductases, otherwise named aldo-keto reductases [7], and share common features such as monomeric structure (30–40 kDa), cytosolic subcellular localization and a cosubstrate specificity for NADPH. Despite overall similarities they can be distinguished on the basis of substrate specificity and their sensitivity to various inhibitors. Thus, barbiturate-sensitive aldehyde reductases and quercitrin-sensitive ketone reductases have been described [2]. Furthermore, enzymes like NAD(P)H:quinone-oxidoreductase (EC 1.6.99.2), dihydrodiol dehydrogenase (EC 1.3.1.20) and hydroxysteroid dehydrogenases (3α-, 3β-, 17β-) might be involved in the metabolism of carbonyl compounds or, as has been supposed, might even be identical to enzymes described previously as carbonyl reductases [8–13].

However, the number of distinct reductases in a tissue and whether or not they are the same as similar enzymes in other tissues is of particular relevance with regard to the search for the physiological role of these enzymes.

In previous investigations it has been shown that metyrapone serves as a good model substrate for studying reductive carbonyl metabolism [14, 15] because it is almost exclusively reduced at its ketone function rather than being oxidized at the nitrogen of the two pyridine rings which would lead to metyrapone *N*-oxides [16]. It was demonstrated that metyrapone reduction occurs in various permanent cell lines in culture thus emphasizing the essentiality of this metabolic pathway [17]. Kahl [18] first reported the enzymatic reduction of the cytochrome P450 inhibitor to the corresponding alcohol metabolite metyrapol by rat liver microsomes. Accordingly, a membrane bound metyrapone reductase (34 kDa) was purified from mouse liver microsomes [14] and, besides being characterized as aldehyde reductase, was demonstrated as being involved in physiological steroid metabolism [19]. Moreover, structural and functional homologies were found between metyrapone reductase from mouse liver microsomes and 3α-hydroxysteroid dehydrogenase from *P. testosteroni* [20].

In this study the situation in human liver was of interest. We characterized metyrapone reduction in human liver cytosol and microsomes using different cosubstrates as well as diagnostic inhibitors, in particular 5α-dihydrotestosterone, to obtain information on the enzymes which might be involved. In addition we checked human liver metyrapone reducing enzymes for immunological and thus

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structural homologies using the antibody against the purified microsomal mouse liver enzyme in the immunoblot analysis. A preliminary account of this work has been presented [21].

MATERIALS AND METHODS

Materials. Human liver microsomes and cytosol from both sexes were kindly supplied by O. Pelkonen (Oulu, Finland). They had been derived from legal medical biopsies or from the livers of individuals post mortem. Metyrapone was purchased from Fluka AG (Buchs, Switzerland). Enzymatic tests were performed using NADH, $NADP^+$, NADPH, G-6-P and G-6-P-DH from Boehringer Mannheim (Mannheim, F.R.G.). For HPLC, acetonitrile of HPLC-grade from E. Merck (Darmstadt, F.R.G.) was used. For inhibitor studies quercitrin and dicumarol were purchased from Roth (Karlsruhe, F.R.G.); 5α-dihydrotestosterone from Aldrich Chemie (Steinheim, F.R.G.); indomethacin from the Sigma Chemical Co. (St Louis, MO, U.S.A.) and sodium barbitone from E. Merck. Low molecular weight markers were obtained from BioRad (Munich, F.R.G.); sodium dodecyl sulphate (SDS*) from Fluka; acrylamide, methylenebisacrylamide, 4-chloro-1-naphthol and H_2O_2 from E. Merck; nitrocellulose from Schleicher & Schuell (Dassel, F.R.G.) and peroxidase-conjugated anti-rabbit IgG antibodies from Dakopatts (Hamburg, F.R.G.). All other chemicals used in the experiments were reagent grade and were obtained from commercial suppliers.

Preparation of subcellular fractions. Samples of human livers were received within less than 1 hr after medical biopsy or death of the individual and homogenized immediately in 4 vol. of 20 mM Tris–HCl buffer pH 7.4, containing 250 mM sucrose and 1 mM EDTA, using a glass–Teflon Potter–Elvehjem homogenizer. The homogenate was centrifuged at 600 *g* for 10 min to sediment the nuclei and cell debris. The resulting supernatant was centrifuged at 10,000 *g* to sediment mitochondria. The supernatant at this stage was centrifuged at 170,000 *g* for 1 hr to sediment microsomes, finally giving a clear cytoplasmic supernatant. Cytosols were dialysed against 1000 vol. of a 50 mM sodium phosphate buffer pH 7.4, to eliminate physiologically occurring pyridine nucleotides. The microsomal pellet was resuspended in the homogenizing buffer, giving a protein concentration of 20 mg/mL.

SDS–PAGE. SDS–PAGE of microsomal and cytosolic fractions of five individual livers was carried out with a discontinuous system as described by Laemmli [22] using 10% acrylamide in the separating gel. Each of the lanes was loaded with a sample containing 15 μg protein of the respective fraction.

Preparation of antisera. The purification of the microsomal metyrapone reductase from mouse liver, immunization of rabbits and preparation of antisera were carried out as described previously [15].

Immunoblot. Electrophoretically separated proteins were transferred to nitrocellulose sheets [23]. Antisera against the microsomal metyrapone reductase were diluted 1:1,000 and incubated with protein saturated nitrocellulose sheets. Antigen–antibody complexes were detected by peroxidase-conjugated secondary antibodies specific for rabbit IgG (dilution 1:1000). These complexes were visualized by the peroxidase reaction (chloronaphthol method) [24].

Enzyme assay. Assay of metyrapone reduction was performed by preincubating 60 μL of the enzyme solution in 50 mM sodium phosphate buffer pH 7.4. For inhibitor studies, 5 μL of the respective inhibitor were added to a final concentration of 1 mM. Inhibitors which were not sufficiently soluble in buffer were dissolved in ethanol or 0.03 M NaOH. Control velocities were determined in the presence of appropriate quantities of the solvents. After the preincubation period of 3 min the reaction was started by adding 5 μL of metyrapone (final concentration 1 mM, found to be saturating) and 5 μL of the respective cosubstrate (final concentrations: NADH 3.2 mM; NADPH 3.2 mM; NADPH-regenerating system: $NADP^+$ 0.8 mM, G-6-P 6 mM, G-6-P-DH 0.35 units, $MgCl_2$ 3 mM) to a final volume of 75 μL. The reduction was stopped after 30 min by mixing 15 μL of the reaction sample with 45 μL of ice-cold acetonitrile. The samples were centrifuged for 6 min at 8000 *g* in the cold and 20 μL of the supernatant were taken for the determination of metyrapone and metyrapol by HPLC analysis.

Determination of metyrapone and metyrapol by HPLC. Metyrapone and its reduced alcohol metabolite metyrapol were detected on a BioRad reversed phase HPLC system, using an Octadecyl-Si 100 polyol (Serva, Heidelberg, F.R.G.) matrix column (4.5 mm × 25 cm) and an eluent of 30% acetonitrile (v/v) in 30 mM phosphate buffer pH 7.4. Metyrapol elutes at about 6.5 min, metyrapone at 10 min (flow rate: 1 mL/min) and they are monitored at 254 nm with an UV monitor and HPLC integration software (BioRad).

Protein determination. Protein concentration was determined by the method of Lowry *et al.* [25] using bovine serum albumin as standard.

RESULTS

Specific activity and cosubstrate requirement of metyrapone reduction

Figure 1 illustrates the specific activity and the cosubstrate requirement of metyrapone reduction in cytosol and microsomes of human liver. Both subcellular fractions show remarkable rates of metyrapol formation with the cytosolic activities exceeding those of the respective microsomes (>30%) in the case of NADPH or the NADPH-regenerating system as cosubstrate. However, considerable reduction of metyrapone also occurs with NADH. Whereas in dialysed cytosol this accounts for only 14% compared to that occurring with NADPH, it came up to 36% in the microsomal fractions. This may indicate that NADH can serve as an alternative electron donor for metyrapone reduction at least in microsomes.

Inhibition profile of metyrapone reduction

As shown in Fig. 2, cytosolic metyrapone

* Abbreviations: SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis.

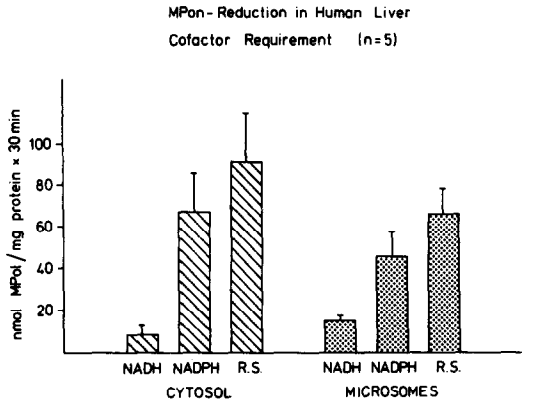

Fig. 1. Reduction of metyrapone (MPon) in mouse liver cytosol and microsomes with different cosubstrates. The specific activity was assayed in the standard reaction mixture containing 50 mM sodium phosphate buffer pH 7.4, 1 mM metyrapone and either 3.2 mM NADH, 3.2 mM NADPH or a NADPH-regenerating system (R.S.). Cytosols were dialysed prior to the enzyme assay against 1000 vol. of 50 mM sodium phosphate buffer, pH 7.4, to eliminate physiologically occurring pyridine nucleotides. Activity is expressed as nmol/mg protein metyrapol (MPol) formed in 30 min. Values are means ± SD of five individual livers.

reduction was moderately inhibited by dicumarol, the characteristic inhibitor of NAD(P)H:quinone-oxidoreductase (formerly called DT-diaphorase) [26], and indomethacin, the specific inhibitor of dihydrodiol dehydrogenase [10], with a residual enzyme activity of 66 and 72%, respectively. No sensitivity to the flavonoid quercitrin or to barbiturates was detected in this fraction.

In microsomes metyrapol formation was likewise moderately decreased by dicumarol (70% of the uninhibited enzyme activity), whereas quercitrin, indomethacin and barbiturate caused only a weak inhibition (78, 84 and 83%, respectively).

In contrast, 5α-dihydrotestosterone, the active form of the androgen steroid testosterone, very strongly inhibited metyrapone reduction in the microsomal fractions (36% residual enzyme activity), whereas in cytosol it has almost no effect on metyrapol formation (86% residual enzyme activity) (Fig. 3).

Immunoblot analysis

Microsomal and cytosolic proteins of the five human livers were electrophoretically separated (15 μg of protein on each lane) and subjected to an immunoblot analysis. Polyclonal antibodies raised in rabbits against the 34 kDa metyrapone reductase from mouse liver microsomes cross-reacted specifically with the homologous protein in human liver microsomes in the same molecular weight region (Fig. 4), and did this in the same way as with the respective mouse enzyme against which they were originally raised [15]. These common antigenic determinants point to structural homologies between the microsomal metyrapone reductases in mouse and human liver.

No cross-reaction occurred with any protein of the respective cytoplasmic fractions indicating the absence of common antigenic determinants.

DISCUSSION

Metyrapone reducing activities were found in both human liver cytosol and microsomes. The enzyme activities exceeded those in mouse liver cytosol and

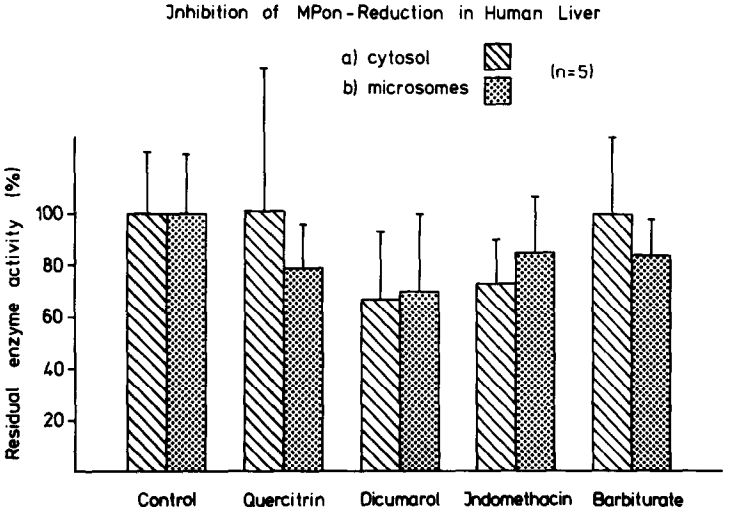

Fig. 2. Effects of inhibitors on metyrapone reduction in mouse liver cytosol and microsomes. The enzyme activity was assayed in the standard reaction mixture containing 50 mM sodium phosphate buffer pH 7.4, 1 mM metyrapone, a NADPH-regenerating system and 1 mM inhibitor. The percentages are calculated from uninhibited control experiments. Values are means ± SD of five individual livers.

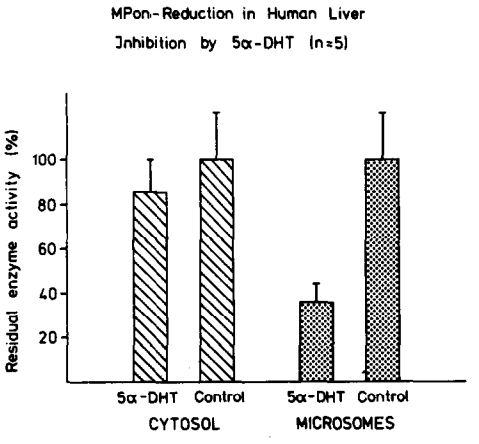

Fig. 3. Effect of 5α-dihydrotestosterone (5α-DHT) on metyrapone reduction in mouse liver cytosol and microsomes. The enzyme activity was assayed in the standard reaction mixture containing 50 mM sodium phosphate buffer pH 7.4, 1 mM metyrapone, a NADPH-regenerating system and 1 mM 5α-dihydrotestosterone where applicable. The percentages are calculated from uninhibited control experiments. Values are means ± SD of five individual livers.

microsomes, found in earlier studies [14], 3.6-fold. However, concerning cosubstrate dependence, mouse [14] and human liver metyrapone reductases seem to be very similar: whereas metyrapol formation in cytosol shows a strong preference for NADPH that in the microsomes obviously occurs with NADH also.

Carbonyl reducing enzymes are in principle cytosolic, monomeric (30–40 kDa), NADPH-dependent oxidoreductases and were originally subclassified into barbiturate-sensitive aldehyde reductases and quercitrin-sensitive ketone reductases [2]. In addition to these classic carbonyl reductases other enzymes like dihydrodiol dehydrogenase (EC 1.3.1.20), NAD(P)H:quinone-oxidoreductase (EC 1.6.99.2) and hydroxysteroid dehydrogenases (3α-, 3β- and 17β-) were described as being involved in carbonyl reduction or as even being identical to enzymes described previously as carbonyl reductases [8–13]. Investigations on the sensitivity of metyrapone reducing enzymes to diagnostic inhibitors have shown that in mouse liver microsomes metyrapone is reduced by an aldehyde reductase [14] and in the respective cytosol by a ketone reductase [15]. Moreover, the microsomal enzyme was purified to homogeneity, showing a molecular weight of 34 kDa [14].

In this study the enzyme activity in both fractions is not affected by barbiturate or quercitrin.

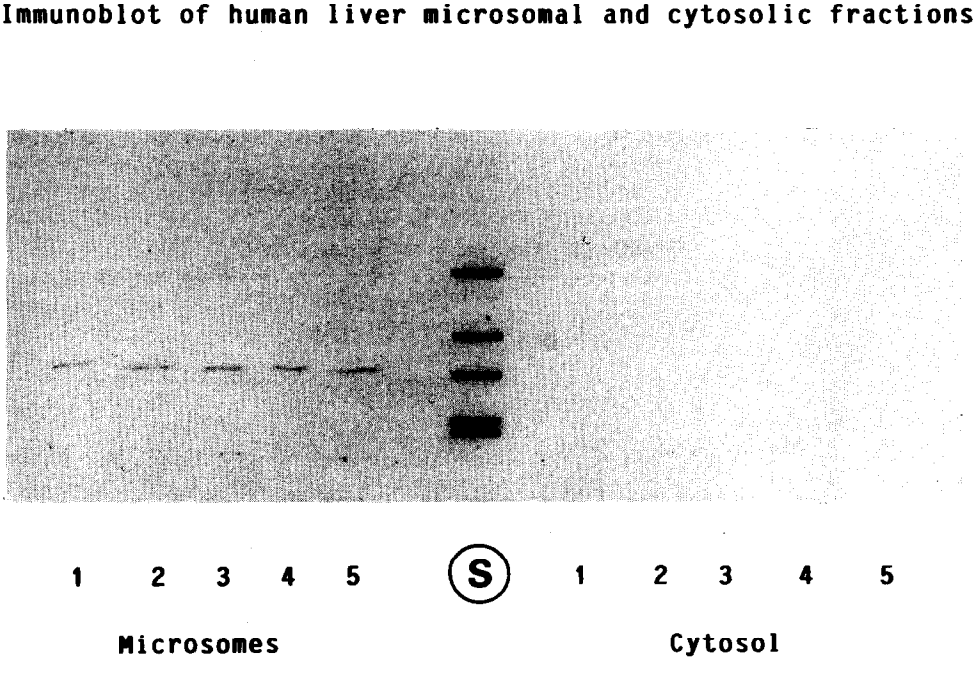

Fig. 4. Immunoblot of microsomal and cytosolic fractions of five individual human livers after SDS–PAGE. Polyclonal antibodies raised in rabbits against the microsomal metyrapone reductase from mouse liver were incubated as primary antibody. Antigen–antibody complexes were identified by the peroxidase reaction of the secondary conjugated antibody specific for rabbit IgG. Positive cross-reaction was found with the homologous protein of the microsomal fractions in the 34 kDa molecular weight region. (S = molecular weight standard: albumin, bovine = 66 kDa; albumin, egg = 45 kDa; glyceraldehyde-3-phosphate dehydrogenase = 36 kDa; carboanhydrase = 29 kDa; trypsinogen = 24 kDa).

Accordingly, following the classic inhibitor subclassification of carbonyl reductases in human liver metyrapone is neither reduced by aldehyde reductase or by ketone reductase. In cytosol dicumarol, the potent inhibitor of NAD(P)H:quinone-oxidoreductase [26], and indomethacin, the inhibitor of dihydrodiol dehydrogenase [10], moderately decreased metyrapol formation, pointing to a possible participation of the respective enzymes in this reaction.

In human liver microsomes part of the reduction of metyrapone seems likewise to be mediated by NAD(P)H:quinone-oxidoreductase because of a considerable inhibition by dicumarol [26]. Surprisingly, metyrapol formation in this fraction is strongly inhibited by 5α-dihydrotestosterone, the active form of the androgen steroid testosterone. 5α-Dihydrotestosterone, therefore, might be the physiological substrate for the microsomal metyrapone reductase. This would resemble the situation in mouse liver where metyrapol formation via microsomal aldehyde reductase was inhibited by steroids, suggesting the enzyme to be involved in physiological steroid metabolism [14]. This was finally confirmed with purified metyrapone reductase in the case of androsterone oxidation [19, 20].

For estimating immunological and thus structural homologies between mouse and human liver microsomal metyrapone reductases, polyclonal antibodies were raised against the purified mouse liver enzyme. Applying the immunoblot technique the antibodies cross-reacted with only one single protein band of human liver microsomes corresponding to the mouse liver enzyme in the 34 kDa molecular weight region. These common antigenic determinants point to structural homologies between the microsomal metyrapone reductases of mouse and human liver origin.

Structural as well as functional relationships between 3α-hydroxysteroid dehydrogenase from *P. testosteroni* and metyrapone reductase from mouse liver microsomes were found in previous investigations [19, 20] where it was demonstrated that the enzymes share features such as substrate specificity for the xenobiotic metyrapone and the steroid androsterone; dual cosubstrate specificity for NADPH and NADH; and common antigenic determinants as revealed by immunoblot analysis.

However, further work on the carbonyl reducing enzymes, particularly comparative biochemical studies in connection with steroid metabolism, is needed in order to understand fully the function of these enzymes.

In conclusion, metyrapone, the diagnostic cytochrome P450 inhibitor, is reduced at its ketone function to the corresponding alcohol metabolite metyrapol in human liver cytosol and microsomes. The reaction is mediated by carbonyl reductases which do not follow the classic inhibitor subclassification of this enzyme family. NADPH is the more potent cosubstrate in both fractions, although considerable enzyme activity in microsomes occurs also with NADH. In addition to the functional resemblances concerning cosubstrate dependence and substrate specificity there also seem to exist structural homologies between microsomal metyrapone reducing enzymes from mouse and human liver, and 5α-dihydrotestosterone is postulated to be the physiological substrate of the microsomal metyrapone reductase from human liver.

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REFERENCES

1. Ziegler DM, Detoxication: oxidation and reduction. In: *The Liver: Biology and Pathobiology* (Eds. Arias IM, Jakoby WB, Popper H, Schachter D and Shafritz DA), pp. 363–374. Raven Press, New York, 1988.
2. Felsted RL and Bachur NR, Mammalian carbonyl reductases. *Drug Metab Rev* **11**: 1–60, 1980.
3. Iwata N, Inazu N and Satoh T, Changes in rat ovarian carbonyl reductase activity and content during the estrous cycle, and localization. *Biol Reprod* **42**: 161–166, 1990.
4. Smolen A and Anderson AD, Partial purification and characterization of a reduced nicotinamide adenin dinucleotide phosphate-linked aldehyde reductase from rat heart. *Biochem Pharmacol* **25**: 317–323, 1976.
5. Pietruszko R and Chen FF, Aldehyde reductase from rat liver is a 3α-hydroxysteroid dehydrogenase. *Biochem Pharmacol* **25**: 2721–2725, 1976.
6. Ikeda M, Hattori H and Ohmori S, Properties of NADPH-dependent carbonyl reductases in rat liver cytosol. *Biochem Pharmacol* **33**: 3957–3961, 1984.
7. Wermuth B, Aldo-keto reductases. In: *Enzymology of Carbonyl Metabolism* (Eds. Flynn TG and Weiner H), Vol. 2, pp. 209–230. Alan R. Liss, New York, 1985.
8. Sawada H, Hara A, Nakayama T, Nakagawa M, Inoue Y, Hasebe K and Zhang Y, Mouse liver dihydrodiol dehydrogenases: identity of the predominant and a minor form with 17β-hydroxysteroid dehydrogenase and aldehyde reductase. *Biochem Pharmacol* **37**: 453–458, 1988.
9. Nakagawa M, Tsukada F, Nakayama T, Matsuura K, Hara A and Sawada H, Identification of two dihydrodiol dehydrogenases associated with 3(17)α-hydroxysteroid dehydrogenase activity in mouse kidney. *J Biochem* **106**: 633–638, 1989.
10. Penning TM, Mukharji I, Barrows S and Talalay P, Purification and properties of a 3α-hydroxysteroid dehydrogenase of rat liver cytosol and its inhibition by anti-inflammatory drugs. *Biochem J* **222**: 601–611, 1984.
11. Hara A, Taniguchi H, Nakayama T and Sawada H, Purification and properties of multiple forms of dihydrodiol dehydrogenase from human liver. *J Biochem* **108**: 250–254, 1990.
12. Inazu N, Inaba N and Satoh T, Localization and regulation of ovarian carbonyl reductase in rats. In: *Enzymology and Molecular Biology of Carbonyl Metabolism* (Eds. Weiner H and Flynn TG), Vol. 2, pp. 323–334. Alan R. Liss, New York, 1989.
13. Hara A, Usui S, Hayashibara M, Horiuchi T, Nakayama T and Sawada H, Microsomal carbonyl reductase in rat liver. Sex difference, hormonal regulation and characterization. In: *Enzymology and Molecular Biology of Carbonyl Metabolism* (Eds. Weiner H and Flynn TG), pp. 401–414. Alan R. Liss, New York, 1987.
14. Maser E and Netter KJ, Purification and properties of a metyrapone reducing enzyme from mouse liver

microsomes—this ketone is reduced by an aldehyde reductase. *Biochem Pharmacol* **38**: 3049–3054, 1989.
15. Maser E and Netter KJ, Reductive metabolism of metyrapone by a quercitrin-sensitive ketone reductase in mouse liver cytosol. *Biochem Pharmacol* **41**: 1595–1599, 1991.
16. DeGraeve J, Gielen JE, Kahl GF, Tüttenberg KH, Kahl R and Maume B, Formation of two metyrapone *N*-oxides by rat liver microsomes. *Drug Metab Dispos* **7**: 166–170, 1979.
17. Gebel T, Maser E and Netter KJ, The occurrence of carbonyl reduction in continuous cell lines emphasizes the essentiality of this metabolic pathway. *FEBS Lett* **282**: 359–362, 1991.
18. Kahl GF, Experiments on the metyrapone reducing microsomal enzyme system. *Naunyn Schmiedebergs Arch Pharmacol* **266**: 61–74, 1970.
19. Maser E, Oppermann U and Netter KJ, Structural and functional relationships between carbonyl reducing enzymes from mammalian liver microsomes and 3α-hydroxysteroid dehydrogenase from *Pseudomonas testosteroni*. *FEBS Lett,* submitted.
20. Maser E, Oppermann U and Netter KJ, Immunological homologies between carbonyl reducing enzymes from procaryonts and mammalian liver microsomes. *Eur J Pharmacol* **183**: 1366, 1990.
21. Maser E and Gebel T, Reductive metabolism of metyrapone in human liver microsomes and cytosol. *Naunyn Schmiedebergs Arch Pharmacol* **343** (Suppl): R10, 1991.
22. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacterophage T4. *Nature* **227**: 680–685, 1970.
23. Kyhse-Anderson J, Electroblotting of multiple gels. A simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide gels to nitrocellulose. *J Biochem Biophys Methods* **10**: 203–209, 1984.
24. Hawkes R, Niday E and Gordon J, A dot immunobinding assay for monoclonal and other antibodies. *Anal Biochem* **119**: 142–147, 1982.
25. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
26. Lind C, Hochstein P and Ernster L, DT-diaphorase as a quinone reductase: a cellular control device against semiquinone and superoxide radical formation. *Arch Biochem Biophys* **216**: 178–185, 1982.