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PURIFICATION AND PROPERTIES OF A METYRAPONE-REDUCING ENZYME FROM MOUSE LIVER MICROSOMES—THIS KETONE IS REDUCED BY AN ALDEHYDE REDUCTASE

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Abstract—A ketone reducing enzyme was purified to homogeneity from female mouse liver microsomes, using the diagnostic cytochrome P-450 inhibitor metyrapone as a substrate. In contrast to the usually employed indirect spectrophotometric recording of pyridine nucleotide oxidation at 340 nm, a HPLC method was applied for direct alcohol metabolite determination. Purification of the carbonyl reductase resulted in a 360-fold increase in specific activity together with a single band in the 34 kD region after SDS–polyacrylamide gel electrophoresis. Phenobarbital, indomethacin, dicoumarol and 5α-dihydrotestosterone inhibited the enzyme, whereas quercitrin did not affect the enzyme activity. Thus, by inhibitor classification of carbonyl reductases the ketone metyrapone is reduced by an aldehyde reductase, rather than by a ketone reductase. Dihydrotestosterone, the strongest inhibitor, is supposed to be the physiological substrate for the purified enzyme. It was demonstrated that during the steps of purification both NADPH and NADH can supply the required reducing equivalents, although the activity with NADH is weaker. The highest activity was obtained using an NADPH-regenerating system. Ethanol and the nonionic detergent Emulgen 913 led to an increased specific activity, indicating that the enzyme is bound to the membranes of the endoplasmic reticulum in a latent state. From these results it is concluded that the microsomal metyrapone-reducing enzyme belongs to the family of carbonyl reductases, but differs from the common patterns of their classification with regard to cofactor requirement and inhibitor susceptibility.

Xenobiotic compounds bearing a carbonyl function are widely distributed in nature and are ingested by humans through nutritional and therapeutical routes. In addition, several non-carbonyl compounds are metabolized to aldehydes and ketones by oxidative deamination *in vivo*. Most of these substances undergo reduction of the carbonyl group prior to their elimination as alcohol products or as the respective conjugates. The enzymes responsible for these biotransformations are called carbonyl reductases, which share typical features such as: monomeric structure with relatively low molecular weight (30–40 kD), dependence on NADPH as cofactor, localization in the cytoplasm and insensitivity to pyrazole, the potent inhibitor of alcohol dehydrogenase. They are divided into two classes, aldehyde reductases and ketone reductases, based on their substrate specificity and inhibitor sensitivity [1]. In general, aldehyde reductases do not reduce ketones (an exception is the ketone daunorubicin, which is reduced by rat liver aldehyde reductase [2]) and are inhibited by barbiturates. In contrast, ketone reductases can reduce many aldehydes, are insensitive to barbiturates, but are inhibited by the flavonoid quercitrin. Aldehyde-ketone reductases occur in multiple forms in animal tissues and several of them have been characterized and purified to apparent homogeneity. Cytosolic forms of carbonyl reductase have been shown to occur in liver of rat [2, 3], rabbit [4, 5] and humans [6–8]; in kidney of rat [7], rabbit [9], pig [10], ox [11] and humans [7]; in bovine brain [13] and that of rat [12] and humans [12, 14], exhibiting a similar broad substrate specificity for exogenous compounds. However, in terms of reduction of physiological substrates they seem to be relatively specific, and are thought to be involved in the metabolism of quinones [11], prostaglandins [14] and steroids [15–17]. Reduction of carbonyl compounds also takes place in membranous fractions of cells, i.e. rabbit liver mitochondria [5] and microsomes of guinea-pig [18, 19] and rat [20] liver.

In previous investigations we found that the diagnostic cytochrome P-450 inhibitor metyrapone [21] is rapidly reduced to the alcohol metabolite metyrapol [22, 23] in female mouse liver microsomes [24]. This study describes the purification of the microsomal metyrapone reducing enzyme from female mouse liver to homogeneity together with its characterization and identification as an enzyme differing from common patterns of carbonyl reductase classification with respect to substrate specificity, cofactor requirement and inhibitor sensitivity. A preliminary account of this work has been presented [25].

MATERIALS AND METHODS

Animals. Female NMRI mice (25–30 g) were used for the experiments.

Chemicals. Metyrapone was purchased from Fluka

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AG (Buchs, Switzerland). Enzymatic tests were performed using NADH, NADP, NADPH, G6P and G6P-DH from Boehringer Mannheim (Mannheim, F.R.G.). For HPLC acetonitrile of HPLC-grade from E. Merck (Darmstadt, F.R.G.) was used. Enzyme purification was carried out with octyl-sepharose CL-4B and CM-sepharose CL-6B from Pharmacia (Freiburg, F.R.G), DEAE-cellulose from E. Merck (Darmstadt, F.R.G.) and hydroxyapatite SC from Serva (Heidelberg, F.R.G.) The detergents listed below were supplied from the following companies: Emulgen 913 (Kao-Atlas Co., Tokyo, Japan), sodium cholate and Triton X-100 (E. Merck, Darmstadt, F.R.G.). For inhibitor studies quercitrin and dicoumarol were purchased from Roth (Karlsruhe, F.R.G.), phenobarbital and pyrazole from Fluka (Neu-Ulm, F.R.G.) and 5α-dihydrotestosterone from Aldrich-Chemie (Steinheim, F.R.G.). The low molecular weight markers were obtained from Sigma (Deisenhofen, F.R.G.). All other chemicals used in the experiments were reagent grade and were obtained from commercial suppliers.

Preparation of liver microsomes. Liver microsomes were prepared according to Netter [26]. After perfusing the livers with an ice-cold isotonic solution of KCl they were homogenized in 4 vol. of 20 mM Tris–HCl buffer, pH 7.4, containing 250 mM sucrose, 1 mM EDTA, 1 mM phenylmethanesulfonylfluoride (PMSF). After centrifugation (105,000 *g*) the microsomal pellet was washed once with 0.15 M KCl to remove glycogen and other proteins and then resuspended in the homogenization buffer without PMSF.

Purification of the enzyme. The microsomal suspension (about 20 mg of protein/ml was diluted with an equal volume of a 10 mM sodium phosphate buffer, pH 7.2, containing 1 mM EDTA, 1 M NaCl, 40% glycerol (w/v) and 0.4% (w/v) of the nonionic detergent Emulgen 913. The solution was gently stirred for 30 min and subsequently centrifuged at 218,000 *g* for 45 min. The supernatant was adjusted to 0.4% (w/v) of sodium cholate before being applied to the octyl-sepharose CL-4B column.

In order to separate the metyrapone reductase from cytochrome P-450 and cytochrome P-450 reductase a method according to Kling *et al.* [27] was performed. The following buffers were used: Buffer A: 10 mM sodium phosphate, 1 mM EDTA, 500 mM NaCl, 20% (w/v) glycerol, 0.5% (w/v) sodium cholate, pH 7.4; Buffer B: 10 mM sodium phosphate, 1 mM EDTA, 400 mM NaCl, 20% (w/v) glycerol, 0.4% (w/v) sodium cholate, 0.1% (w/v) Emulgen 913, pH 7.4; Buffer C: 10 mM sodium phosphate, 1 mM EDTA, 20% (w/v) glycerol, 2% (w/v) Emulgen 913, pH 7.4.

Solubilized microsomes were applied to the octyl-sepharose CL-4B column (1.6 × 20 cm) previously equilibrated with 300 ml of buffer A. Elution was performed with buffer A until the end of peak 2, then with buffer B until the end of peak 3 followed by buffer C eluting peak 4.

The elution profile was monitored measuring the absorbance of the fractions at the wavelength of 417 nm. The column flow rate was 50 ml/hr and the volume per fraction 5 ml.

The metyrapone reductase activity coincides only with peak 3, the fractions of which were collected, concentrated through an Amicon PM-10 membrane and dialysed against 5 mM sodium phosphate buffer, pH 7.4.

The dialysed enzyme solution was applied to a column (1.6 × 20 cm) packed with DEAE-cellulose, equilibrated with 5 mM sodium phosphate buffer, pH 7.4. The column was washed with the equilibration buffer and the adsorbed carbonyl reductase was eluted with a 30 mM phosphate buffer, pH 7.4, at a flow rate of 30 ml/hr. Enzymatically active fractions were directly applied on a hydroxyapatite column (1.6 × 5 cm), equilibrated with a 5 mM phosphate buffer, pH 7.4. The column was rinsed with the equilibration buffer, then with a 150 mM phosphate buffer, pH 7.4. The enzyme was eluted with the 150 mM phosphate buffer, pH 7.4, containing 0.1% Triton X-100. The fractions with high enzyme activity were pooled, concentrated through an Amicon PM-10 membrane and dialysed against 5 mM sodium phosphate buffer, pH 6.6.

The dialysed pool was loaded onto a column (1.6 × 20 cm) of CM-sepharose equilibrated with 5 mM phosphate buffer, pH 6.6. The column was washed with the equilibration buffer, then step by step with 20 mM, 40 mM and 60 mM phosphate buffer, pH 6.6. The enzyme was eluted with a 60 mM phosphate buffer, pH 7.4. Throughout the purification, the temperature was kept at 4°.

Enzyme assay. Assay of ketone reductase was performed by preincubating 100 μl of enzyme solution in 50 mM sodium phosphate buffer, pH 7.4. For inhibitor studies 10 μl of the respective inhibitor were added to a final concentration of 1 mM. In corresponding experiments with 5α-dihydrotestosterone, the concentrations ranged from 0.05 mM to 1.6 mM. Inhibitors which were not sufficiently soluble in buffer were dissolved in ethanol or 0.04 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 10 μl of metyrapone (final concentration 4.6 mM) and 10 μl of the respective cosubstrate (final concentrations: NADH 3.2 mM; NADPH 3.2 mM; NADPH-regenerating system: NADP 0.8 mM, G6P 6 mM, G6P-DH 0.35 U, $MgCl_2$ 3 mM) to a final volume of 150 μl. The reduction was stopped by mixing 50 μl of the reaction sample with 150 μl of ice-cold acetonitrile. The samples were centrifuged for 6 min at 8000 *g* in the cold and 20 μl of the supernatant served for the determination of metyrapone and metyrapol by HPLC-analysis as described previously [23].

SDS–polyacrylamide gel electrophoresis. Sodium dodecylsulphatepolyacrylamide gel electrophoresis was carried out as described by Laemmli [28] using 10% acrylamide in the separating gel. Protein bands were visualized applying the silver stain technique according to Ansorge [29].

Protein determination. Protein concentration was determined by the method of Lowry *et al.* [30] or Bradford [31] using bovine serum albumin as standard.

RESULTS

Table 1 summarizes the purification procedure of

Table 1. Purification of microsomal carbonyl reductase

Step	Total protein (mg)	Total activity* (μmol/30 min)	Specific activity (μmol/30 min/mg)	Recovery (%)
Microsomes	960.0	15.36	0.016	100
Solubilized† microsomes	416.5	33.74	0.081	219.7
Octyl-sepharose CL-4B	78.9	25.41	0.322	165.4
DEAE-cellulose	6.9	3.89	0.564	25.3
Hydroxyapatite	2.6	3.12	1.202	20.3
CM-sepharose CL-6B	0.5	2.91	5.824	19.0

* The reductase activity was assayed in the standard reaction mixture containing 50 mM sodium phosphate buffer, pH 7.4, a NADPH-regenerating system and 4.6 mM metyrapone. Activity is expressed as μmol metyrapol formed in 30 min per mg of protein.

† The solubilized microsomal suspension used in this reaction mixture did not contain sodium cholate.

Table 2. Comparison of the specific activity of the microsomal carbonyl reductase using different reducing systems throughout the purification procedure

	Formation of metyrapol* (μmol/30 min/mg)		
	NADH	NADPH	NADPH-reg. sys.
Microsomes	0.005	0.009	0.016
Solubilized† microsomes	0.023	0.036	0.081
Octyl-sepharose CL-4B	0.103	0.238	0.322
DEAE-cellulose	0.259	0.381	0.564
Hydroxyapatite	0.334	1.150	1.202
CM-sepharose CL-6B	1.577	4.018	5.824

* The reductase activity was assayed in the standard reaction mixture containing 50 mM sodium phosphate buffer, pH 7.4, 4.6 mM metyrapone and either 3.2 mM NADH, 3.2 mM NADPH or a NADPH-regenerating system, respectively. Activity is expressed as μmol metyrapol formed in 30 min per mg of protein.

† The solubilized microsomal suspension used in this reaction mixture did not contain sodium cholate.

the microsomal metyrapone-reducing enzyme. Solubilization of the microsomal fraction in 0.2% (w/v) Emulgen 913 already resulted in a 5-fold increase of metyrapol formation, indicating that the carbonyl reductase is bound to the membranes of the endoplasmic reticulum in a latent state. Also each of the following steps resulted in a several-fold increase in specific activity. With a NADPH-regenerating system as electron supply it is enhanced to about 360-fold compared to that in microsomes.

Table 2 compares the specific activity of the enzyme using either NADH, NADPH or a NADPH-regenerating system as electron donors. The ratio of activity of the different electron donors remains nearly constant throughout the purification procedure. From this it becomes obvious that either NADPH or NADH can deliver the required reducing equivalents, although reduction is weaker with NADH. Highest activity is obtained with the NADPH-regenerating system. Incubations containing both cofactors at the same time reveal that there is no synergistic effect of NADH and NADPH.

A sample taken from the various purification steps was subjected to SDS–polyacrylamide gel electrophoresis and subsequent silver stain, as shown in Fig. 1. Lane 1 represents the total quantity of microsomal protein, which was markedly decreased after affinity chromatography on an octyl-sepharose CL-4B column (lane 2). Further purification was achieved with DEAE-cellulose (lane 3), hydroxyapatite (lane 4) and CM-sepharose (lane 5).

The last step of the purification procedure yielded a single band in the 34 kD region as compared to the protein standard, indicating that the microsomal metyrapone reductase was purified to homogeneity.

Table 3 lists some compounds which were tested as inhibitors of the carbonyl reductase in intact microsomes. Dicoumarol, which is known to inhibit NADP(H):quinone oxidoreductase (EC 1.6.99.2, DT-diaphorase), decreased metyrapone reduction to

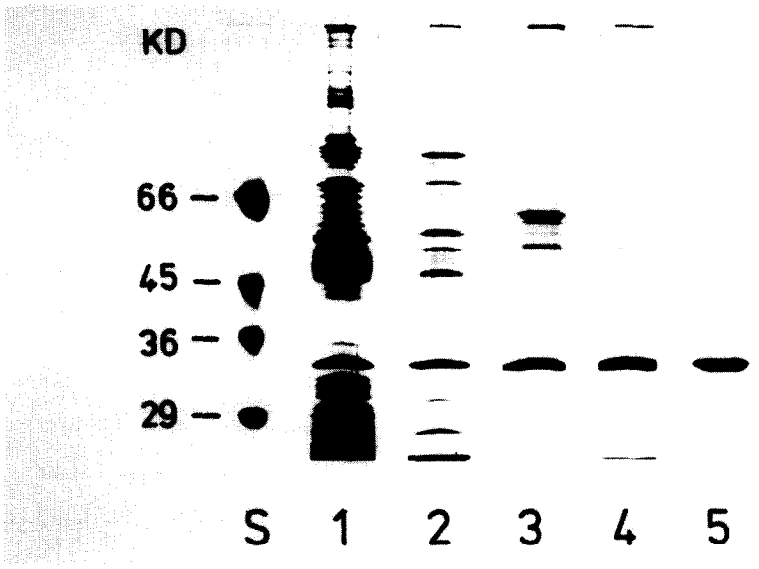

Fig. 1. SDS–polyacrylamide gel electrophoresis of the purification steps of the microsomal metyrapone reductase. Lane S = molecular mass standards (values in kD on the left); lane 1 = protein profile of mouse liver microsomes; lane 2 = after octyl-sepharose; lane 3 = after DEAE-cellulose; lane 4 after hydroxyapatite; lane 5 = after CM-sepharose. Visualizing of protein bands was carried out by the silver stain technique. For details see Materials and Methods.

Table 3. Effects of inhibitors on microsomal metyrapone reductase

Inhibitor	Concentration (mM)	Relative enzyme* activity (%)
None	—	100
Dicoumarol	1	33
Indomethacin	1	52
Phenobarbital	1	56
5α-DHT†	1	29
Pyrazole	1	86
Quercitrin	1	100

* The enzyme activity was assayed in the standard reaction mixture containing 50 mM sodium phosphate buffer, pH 7.4, 4.6 mM metyrapone, a NADPH-regenerating system and inhibitor concentrations of 1 mM, respectively. The percentages are calculated from uninhibited control experiments.

† 5α-DHT; 5α-dihydrotestosterone.

about 33%. Phenobarbital, an inhibitor of aldehyde reductase, and indomethacin also affected metyrapol formation and lowered it to about 56 and 52%, respectively. 5α-Dihydrotestosterone decreased metyrapone reduction to about 29% compared to the uninhibited control experiments and is therefore postulated to be the physiological substrate of this microsomal carbonyl reductase. Increasing concentrations of 5α-dihydrotestosterone (0.05–1.6 mM) led to a linear decrease of enzyme activity using an exponential scale (Fig. 2). Pyrazole, a potent inhibitor of alcohol dehydrogenase had only little effect on metyrapol formation, whereas quercitrin, a flavonoid, showed no inhibition.

However, in the presence of 20% ethanol metyrapone reduction was enhanced to about 157% compared to respective control experiments (data not shown).

DISCUSSION

To date only few membrane-associated carbonyl reductases have been isolated, i.e. from guinea-pig and rat liver microsomes [19, 32, 33]. The objective of these investigations was the purification and characterization of a microsomal carbonyl reductase from mouse liver. The ketone metyrapone proved a useful substrate, since it was shown to be mainly reduced *in vivo* to the respective alcohol, rather than being oxidized at the nitrogens of the two pyridine rings [22, 23, 34]. Moreover, the method of HPLC analysis for direct determination of the product metyrapol is clearly advantageous in comparison to the indirect spectrophotometric recording of pyridine nucleotide oxidation at 340 nm usually employed.

As shown in the results, the specific activity was enhanced about 360-fold after the purification, using a NADPH-regenerating system as electron supply. SDS–polyacrylamide gel electrophoresis of the respective enzyme preparations during the purification procedure gave, after the last step, a single band in the 34 kD region, indicating that the reductase was purified to homogeneity. The molecular weight of the metyrapone reductase is comparable to the values of carbonyl reductases purified by other workers from cytosol [4, 5, 8, 35] or from microsomes [19, 32, 33], although there exist some reports of higher molecular weight forms [8, 19].

Carbonyl reductases have been shown to be dependent on NADPH as a electron donating system [3–6, 14, 33, 35–37]. In this study metyrapol formation occurred with either NADPH or NADH as cofactor, although activity with NADH was weaker. Highest

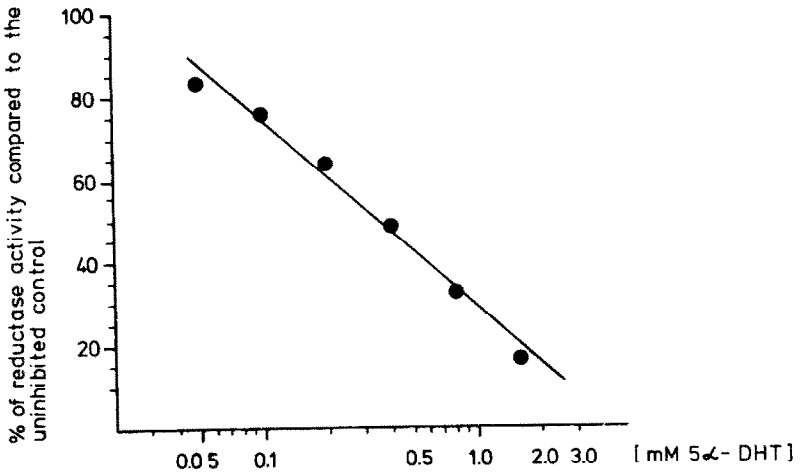

Fig. 2. Inhibition of metyrapone reduction in mouse liver microsomes by various concentrations of 5α-dihydrotestosterone (5α-DHT). The enzyme activity was assayed in the standard reaction mixture containing 50 mM sodium phosphate buffer, pH 7.4, 4.6 mM metyrapone, a NADPH-regenerating system and 5α-DHT concentrations ranging from 0.05 to 1.6 mM. The percentages are calculated from uninhibited control experiments.

activity was obtained with a NADPH-regenerating system. The ratio of the different cosubstrates remains nearly constant throughout the chromatographic steps of purification, providing evidence that the purified enzyme can use both NADPH or NADH. The potency of dual cofactor exploitation was reported until now only of cytosolic carbonyl reductases [12, 15, 38–40].

Inhibitor studies showed that the flavonoid quercitrin, which was designated as an inhibitor of ketone reductases [1] did not inhibit metyrapol formation. In contrast, phenobarbital, which was defined as an inhibitor of aldehyde reductases, decreased the enzyme activity to about 56%. Thus, based on the subdivision of carbonyl reductases with regard to inhibitor susceptibility, metyrapone as a substrate bearing a ketone function is in reality reduced by an aldehyde reductase, rather than by a ketone reductase. These findings are in agreement with Ahmed *et al.* [41], who purified an aldehyde reductase from guinea-pig liver microsomes, which in addition to a variety of aldehydes was capable of reducing the ketone daunorubicin as well. By definition aldehyde reductases do not reduce ketones [1] and the microsomal metyrapone reductase seems to be another exception to this rule.

5α-Dihydrotestosterone proved to be the strongest inhibitor of the metyrapone reductase, which led to the suggestion that dihydrotestosterone is the physiological substrate for this enzyme. This corresponds to other studies [3, 15, 16, 19, 33], which provided evidence that carbonyl reductases are involved in the metabolism of steroids. Other inhibitors of metyrapone reduction were indomethacin and dicoumarol, with the latter being an inhibitor of the DT-diaphorase. Pyrazole, the specific inhibitor of alcohol dehydrogenase did not decrease metyrapol formation.

However, the increasing activity of the carbonyl reductase in the presence of ethanol might be interpreted as a delipidation effect, which was already reported by Sawada *et al.* [32]. They observed an activity enhancement of their membrane-associated carbonyl reductase in the presence of acetone and supposed surrounding membrane lipids to act as endogenous inhibitors on the enzyme. This theory may be confirmed by the fact that solubilization of the microsomal reductase with a nonionic detergent led to a 4–5-fold enhancement of specific activity.

In conclusion it is stated that we purified and characterized a ketone reducing enzyme from mouse liver microsomes, which differs in cofactor requirement and inhibitor susceptibility from common patterns of carbonyl reductase classification.

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