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QUINONE REDUCTION AND REDOX CYCLING CATALYSED BY PURIFIED RAT LIVER DIHYDRODIOL/ 3α-HYDROXYSTEROID DEHYDROGENASE

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Abstract—A highly active preparation of rat liver dihydrodiol/3α-hydroxysteroid dehydrogenase was obtained using a newly developed, rapid purification scheme involving affinity chromatography on Red Sepharose. Depending on the coenzyme present, the purified enzyme was found to catalyse the oxidation of dihydrodiols and steroids or the reduction of substrates with carbonyl or quinone moieties. Using a wide range of synthetic quinones derived from polycyclic aromatic hydrocarbons (PAHs), we observed a pronounced regioselectivity of the quinone reductase activity. Good substrates were the *o*-quinones of phenanthrene, benz(*a*)anthracene, chrysene and benzo(*a*)pyrene with the quinonoid moiety in the K-region which were reduced at rates of 1–10 μmol/min·mg enzyme. 1,4-Benzoquinone, naphthalene-1,2-quinone and benz(*a*)anthracene-8,9-quinone were also reduced at high rates. In contrast, alkyl-substituted quinones such as duroquinone and menadione were poor substrates for the enzyme. During the enzymatic reduction of several *o*-quinones, but not 1,4-benzoquinone, we observed the oxidation of large amounts of NADPH and the consumption of molecular oxygen which is indicative of a redox-cycling process. Thus, the reduction of quinones of PAHs may lead to a facilitated conjugation and excretion of these highly lipophilic compounds, but may also initiate toxic processes due to the formation of reactive oxygen species.

Dihydrodiol dehydrogenase (DDH†; EC 1.3.1.20) is a cytoplasmic enzyme capable of oxidizing aromatic dihydrodiols to the corresponding catechols in an $NADP^+$-dependent reaction [1]. Purification of the rat liver enzyme has been reported by Vogel *et al.* [2] and by Penning *et al.* [3], and the enzyme has been shown to be identical to 3α-hydroxysteroid dehydrogenase in this organ [3]. In addition to the standard substrate, benzene dihydrodiol(*trans*-5,6-dihydroxy-1,3-cyclohexadiene), purified rat liver DDH has been found to oxidize several polycyclic aromatic dihydrodiols [4–6]. Systematic studies with a wide range of polycyclic compounds led to the conclusion that rat liver DDH preferentially oxidizes M-region‡ dihydrodiols such as benz(*a*)anthracene-3,4-dihydrodiol or chrysene-1,2-dihydrodiol [7, 8] while K-region dihydrodiols such as benz(*a*)anthracene-5,6-dihydrodiol or chrysene-5,6-dihydrodiol are generally poor substrates of the enzyme.

We have reported previously that, in addition to the oxidation of dihydrodiols, rat liver DDH also catalyses the reduction of quinones in the presence of NADPH as cofactor [9]. The present study is an extension of this work. Using a newly developed, rapid purification scheme, we have purified rat liver DDH to high activity, and we have investigated systematically the substrate specificity of the enzyme with a wide range of PAH quinones. Moreover, we report the occurrence of potentially toxic redox cycles which are associated with quinone reduction by rat liver DDH.

MATERIALS AND METHODS

Chemicals

Benzene dihydrodiol was synthesized as described [10]. Phenanthrene-1,2- and -3,4-quinones, benz(*a*)anthracene-1,2- and -8,9-quinones, as well as benzo(*a*)pyrene-7,8- and -9,10-quinones were prepared as reported in Ref. 11. Pyrene-1,6- and -1,8-quinones as well as benzo(*a*)pyrene-1,6-, -3,6- and -6,12-quinones were obtained by oxidation of the parent hydrocarbons [12]. The synthesis of naphthalene-1,2-quinone [13], phenanthrene-9,10-quinone [14], benz(*a*)anthracene-4,3-quinone [15], benz(*a*)anthracene-5,6-quinone [14], chrysene-5,6-quinone [16] and benzo(*a*)pyrene-4,5-quinone [14] followed published procedures.

Phenanthrene-1,4-quinone was obtained by the Diels–Alder reaction between styrene and 1,4-benzoquinone after recrystallization from *n*-hexane in 13% yield as yellow crystals with m.p. 148–149° (lit. [17] m.p. 152–154°). Chrysene-3,4-quinone was prepared (red crystals, 85% yield, m.p. >185°) from 3,4-dimethoxychrysene [18] after demethylation by boron tribromide and subsequent oxidation of the

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† Abbreviations: DDH, dihydrodiol dehydrogenase; HSDH, hydroxysteroid dehydrogenase; PAH, polycyclic aromatic hydrocarbon; duroquinone, 2,3,5,6-tetramethyl-1,4-benzoquinone; menadione, 2-methyl-naphthalene-1,4-quinone.

‡ The term M-region is used here is a structural sense, i.e. denoting the 3,4-positions of benz(*a*)anthracene and dibenz(*a,h*)anthracene, the 1,2-positions of phenanthrene and chrysene or the 7,8-position of benzo(*a*)pyrene.

resulting catechol by silver carbonate/celite according to Ref. 19.

1,4-Benzoquinone, duroquinone (2,3,5,6-tetramethyl-1,4-benzoquinone), naphthalene-1,4-quinone, menadione (2-methyl-naphthalene-1,4-quinone) and anthracene-9,10-quinone were purchased from Aldrich (Steinheim, F.R.G.).

Chromatography materials were from Pharmacia (Freiburg, F.R.G.). Procion Red H3B was supplied by ICI Chemicals (Frankfurt, F.R.G.). Nicotinamide cofactors were from Boehringer (Mannheim, F.R.G.). All other chemicals were from commercial sources and were supplied in the highest purity available.

Enzyme purification

Male Wistar rats (Savo Co., Kisslegg, F.R.G.) were killed by a blow on the head and bled. The livers were removed, weighed, washed and homogenized (3 × 20 sec) by an Ultra-Turrax in ice-cold 10 mM Tris–HCl buffer, pH 7.4 (3 mL/g tissue) containing 0.2 mM dithiothreitol and 1 mM EDTA. The following protease inhibitors (dissolved in ethanol) were present in the homogenization buffer (final concentrations in parentheses): phenylmethylsulfonyl fluoride (0.1 mM), L-1-tosylamide-2-phenylethyl chloromethyl ketone (0.1 mM), *Nα-p*-tosyl-L-lysine chloromethyl ketone (0.1 mM), benzamidine (1 mM), leupeptin (4 μg/mL), antipain (4 μg/mL) and pepstatin A (4 μg/mL). The homogenates were centrifuged for 20 min at 10,000 *g*, and the supernatant fractions were centrifuged again at 100,000 *g* for 60 min. The supernatants of the second centrifugation constituted the cytosolic fractions and were used for enzyme purification. All chromatographic steps were carried out at 4°.

Affinity chromatography. The cytosolic fractions of 48 rat livers were applied to a column of Red Sepharose (30 × 5 cm) equilibrated with 20 mM Tris–HCl buffer, pH 7.0, containing 20 mM KCl, 0.2 mM dithiothreitol and 1 mM EDTA. The linear flow rate was 10 cm/hr. Red Sepharose was prepared according to Ref. 20 using Procion Red HE3B as ligand. After the application of up to two column volumes of cytosol, the Red Sepharose was washed with 1 column volume of equilibration buffer, and afterwards with equilibration buffer containing 50 mM KCl until the eluate was colourless. Enzymatic activity was eluted with equilibration buffer containing 2 M KCl. The fractions containing enzymatic activity were pooled, additional KCl was added to increase the molarity of the solution by 1 M and the pooled fractions were immediately subjected to a hydrophobic interaction chromatography on phenylsepharose.

Hydrophobic interaction chromatography. The phenylsepharose column (10 × 5 cm) had been equilibrated with 50 mM Tris–HCl buffer, pH 7.0, containing 2 M KCl and 1 mM EDTA. Following the application of the enzyme solution, the column was washed with equilibration buffer (1–2 column volumes) and subsequently eluted with a linear, decreasing salt gradient (gradient volume approx. 3–4 column volumes) at a linear flow rate of 20 cm/hr. Active fractions eluting at the beginning of the gradient were pooled and dialysed for 2 × 4 hr against saturated ammonium sulfate solution containing 10 mM EDTA which was adjusted to neutral pH by concentrated ammonia. Precipitated protein was separated by centrifugation and dissolved in a minimal amount of the equilibration buffer of the following gel chromatography.

Gel chromatography. A column of Sephadex G-75 superfine (100 × 2.5 cm) had been equilibrated with 20 mM potassium phosphate buffer, pH 7.0, containing 80 mM KCl and, after the application of enzyme solution, was eluted with the same buffer. The gel filtration column had been calibrated with dextran blue, hemoglobin, ovalbumin, cytochrome *c* and sodium sulfate. The linear flow rate was 2 cm/hr. The active fractions which eluted as a single, coherent peak with a M_r of approx. 40,000 were pooled and concentrated by ultracentrifugation on PM-10 membranes (Amicon, Düsseldorf, F.R.G.). Final preparations were stored at −20° (after addition of 50% glycerol) or were quick-frozen in liquid nitrogen and stored at −70°.

Other procedures. DDH activity eluting from the columns was quickly visualized by a published procedure [21], with the following modifications. The test mixture contained: 0.1 mL Triton X-100 (1% in water), 0.1 mL nitroblue tetrazolium (2 mg/mL in water, freshly prepared), 2.3 mM $NADP^+$, 1.8 mM benzene dihydrodiol, 0.02 mL DT-diaphorase (Cl. cluyveri, 2.5 mg/mL in 50% glycerol) and 1 M glycine buffer, pH 9.5 (total volume 1 mL). One hundred microliters of the test mixture were incubated with 20 μL of eluate fractions for 1–5 min in the dark on ELISA plates, and DDH activity was indicated by the development of dark blue precipitate.

Protein concentrations were determined by the method of Lowry *et al.* [22]. In the presence of dithiothreitol, the proteins were precipitated prior to analysis by 10% trichloroacetic acid, centrifuged, taken up in 1 N sodium hydroxide and assayed. SDS-PAGE was carried out according to the procedure of Laemmli [23] in 12% polyacrylamide gels. Fixation and staining of the proteins with Coomassie brilliant blue G-250 was done according to Ref. 24.

Enzyme assays

DDH activity. DDH activity was determined spectrophotometrically at 37° by following the NAD(P)H production at 340 nm ($\varepsilon = 6$) 220 $M^{-1} cm^{-1}$) in the presence of benzene dihydrodiol. The standard assay mixture contained 2.3 mM $NADP^+$ and 1.8 mM benzene dihydrodiol (for the concentrations of other substrates, see Tables) in 1 mL of 50 mM Tris–HCl buffer, pH 9.0. The reaction was started by the addition of 20 μL of enzyme solution. In the case of cytosol as enzyme source, the enzymatic reaction was initiated by the addition of substrate and the blank reaction occurring with cytosol plus $NADP^+$ was subtracted. If inhibitors of DDH were tested, enzyme and inhibitor were preincubated for 2 min, and the reaction was started by the addition of substrate. One unit of enzyme activity was defined as the amount of enzyme producing one micromole of NADPH per minute under these conditions.

Quinone reductase activity. The quinone reductase

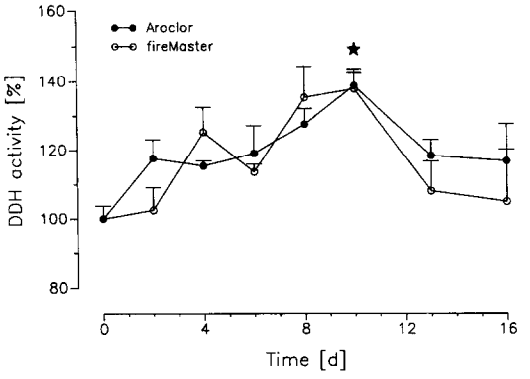

Fig. 1. Induction of DDH activity by polyhalogenated biphenyls. Male Wistar rats were injected intraperitoneally with 500 mg/kg Aroclor 1254 resp. fireMaster BP-6 dissolved in corn oil on day zero [56], and DDH activities were measured on the days indicated in liver cytosol which was prepared as described in Materials and Methods. Data are means ± SEM of three animals. *Significant induction ($P < 0.05$) vs control animals which were treated with corn oil only.

activity of DDH was determined by following the consumption of NADPH at 340 nm. The standard incubation mixture contained 10–20 μg of purified enzyme, 100 μM NADPH and 10–100 μM of quinone substrate (see Table 4) in 50 mM potassium phosphate buffer, pH 7.4. Quinonoid substrates were dissolved in acetonitrile or methanol. Up to 3% of acetonitrile or methanol in the incubation mixture did not affect the enzymatic activity. Buffer, enzyme solution and cofactor were preincubated at 37° for 2 min, and the reaction was started by the addition of substrate. In separate experiments, non-enzymatic reactions between cofactor and quinones were tested and were taken into account when present.

Measurement of oxygen consumption. The consumption of molecular oxygen during the enzymatic reduction of quinones was determined in a Clark-type electrode (BA-3, Eschweiler, Kiel, F.R.G.). Incubations were performed as described for the quinone reductase enzyme assay. In some cases, 10 μg of superoxide dismutase or catalase were added.

RESULTS

Purification of rat liver DDH

Several enzymes of xenobiotic metabolism can be purified with high yield after the application of enzyme inducers to the animals. We have previously reported that DDH activity in rat liver is not induced by common inducers of xenobiotic-metabolizing enzymes such as phenobarbital or 3-methylcholanthrene [9]. Figure 1 shows the result of an induction study using two mixtures of polychlorinated (Aroclor 1254) and polybrominated (fireMaster BP-6) biphenyls with properties of mixed-type inducers [25]. Significant increases in DDH specific activities were noted 10 days after treatment; however, the extent of induction (37 and 38% increase resp.) was too small to make a major contribution to the yield of the enzyme purification. The enzyme used in the present study has therefore been purified from untreated animals.

Compared to previous, rather time-consuming purification schemes of rat liver DDH [2, 3] or 3α-HSDH [26, 27] we here report a rapid three-step procedure which yields a homogeneous enzyme preparation of high activity. The purification as summarized in Table 1 includes a very efficient affinity chromatography step on Red Sepharose, a material which is known to display a high affinity for $NADP^+$-dependent enzymes [20]. Moreover, the sequential combination of columns and the use of a rapid spot-plate technique to identify active fractions (see above) allows the completion of the purification within 3 to 4 days. Figure 2 demonstrates the homogeneity of the final enzyme preparation which displays a M_r on SDS–PAGE of approximately 35,000. It is also apparent that the affinity chromatography is a very efficient step, while the final gel filtration is only necessary to remove minor impurities and salt (ammonium sulfate) from the enzyme solution. The final yield of DDH activity is very high (46%; cf. Table 1).

Catalytic properties of rat liver DDH

The pure enzyme preparation displayed V_{max} and K_m values towards benzene dihydrodiol of 8.58 U/

Table 1. Purification of rat liver DDH

Purification step	Protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification factor
Cytosol	23,400	288	0.012	100	1
Affinity chromatography	610	247	0.405	86	33
Hydrophobic interaction chromatography	56.3	190	3.38	66	275
Gel filtration (final preparation)	34.1	132	3.86	46	314

Cytosols from 48 rats were used for purification as described in Materials and Methods. Enzyme activity was measured in 50 mM Tris–HCl buffer, pH 9.0, in the presence of 2.3 mM $NADP^+$ and 1.8 mM benzene dihydrodiol.

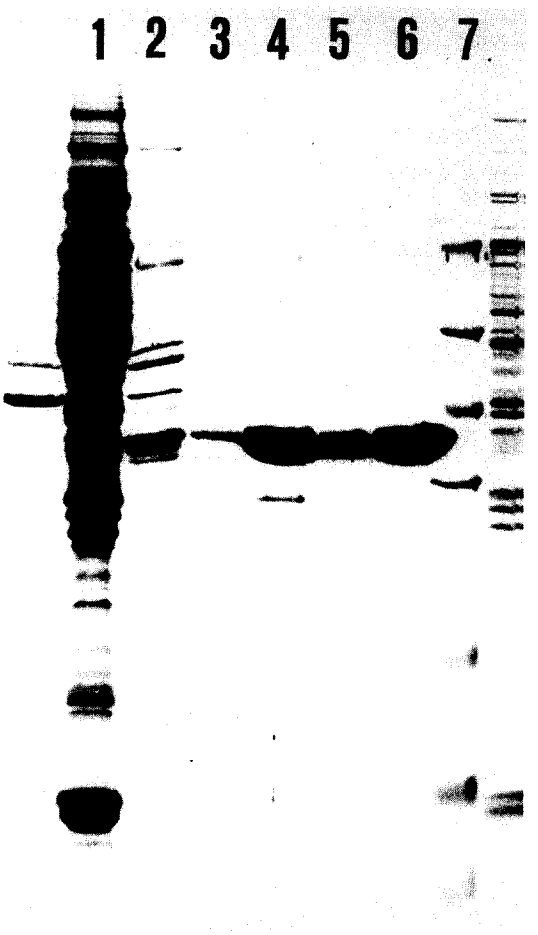

Fig. 2. Purification of rat liver DDH as followed by SDS–PAGE. Lane 1, rat liver cytosol. Lane 2, active fractions after affinity chromatography. Lane 3, active fractions after hydrophobic interaction chromatography. Lane 4, active fractions after ammonium sulfate precipitation. Lanes 5 and 6, final preparation, 2 and 5 μg of protein applied to the gel. Lane 7, standard protein mixture containing bovine serum albumin (68 kD), pyruvate kinase (57 kD), glycerol-3-phosphate dehydrogenase (36 kD), carboanhydrase (30 kD), trypsin inhibitor (21 kD) and cytochrome *c* (14.4 kD).

mg and 2.2 mM, respectively ($k_{cat} = 5.3\ sec^{-1}$). These values are higher than those previously reported [2, 3] which may be due to rapid purification, but also to the presence of dithiothreitol during the chromatographic steps (see below). The substrate specificity of purified rat liver DDH is briefly characterized in Tables 2 and 3. Rat liver DDH is active towards 3α-hydroxysteroids of 5α- and 5β-configuration but does not oxidize 3β-, 17α- or 17β-hydroxysteroids at measurable rates (not shown). The enzyme reduces both *p*-nitrobenzaldehyde and *p*-nitroacetophenone, typical substrates for aldehyde and ketone reductase [28, 29], respectively, but glucuronic acid and glyceraldehyde are reduced at very low rates. The enzyme strongly prefers $NADP^+$ to NAD^+ as a cofactor and also prefers NADPH to NADH during reductive catalysis (not shown). Rat liver DDH is strongly inhibited by 10 μM medroxyprogesterone acetate (by 88%) and 100 μM indomethacine (by 75%) but not by 10 mM phenobarbital, 10 μM Quercetin or 10 mM EDTA (not shown). It is completely inhibited by pre-incubation with *p*-chloromercuribenzoate. This observation is indicative of the presence of a critical SH group in the active centre of the enzyme which should be protected from oxidation during purification by dithiothreitol. The presence of substrate (benzene dihydrodiol) or cofactor delayed the inactivation by *p*-chloromercuribenzoate. We also found that dithiothreitol and antioxidants such as butylated hydroxyanisol increased the enzymatic activity of older, partly inactivated enzyme preparations.

Reduction of quinones by rat liver DDH

Table 4 summarizes the results of a systematic study of the substrate specificity of rat liver DDH towards quinones of mono-, bi- and polycyclic aromatic hydrocarbons of varying structures which were synthesized in our laboratory (see Materials and Methods). In the presence of NADPH, rat liver DDH was found to reduce several quinonoid substrates very efficiently, and in several cases the reaction velocity with quinones was higher than the rate of benzene dihydrodiol oxidation (for comparison, the rate of oxidation of benzene dihydrodiol—measured at pH 7.4 with $NADP^+$ as cofactor—was 4.01 U/mg at 10 mM and 0.21 U/mg at 0.1 mM substrate concentration). The measurement of kinetic constants was not attempted due to the low solubility of most PAH quinones in aqueous media which renders the estimation of available substrate concentrations very difficult. However, the comparison of relative reaction rates reveals a marked preference of the enzyme for quinones of a particular regiospecific substitution. Thus, *p*-quinones are reduced at a rapid rate in the case of the monocyclic 1,4-benzoquinone, at an intermediate rate in the case of the bicyclic naphthalene-1,4-quinone, and are not reduced at all if part of a larger polycyclic ring system (phenanthrene-1,4-quinone, anthracene-9,10-quinone); similarly, quinones in which the quinonoid structure extends over more than one ring such as pyrene-1,6- and -1,8-quinone and benzo(*a*)pyrene-1,6-, -3,6- and -6,12-quinone are very poor substrates of the enzyme. In contrast, several PAH *o*-quinones are good substrates for rat liver DDH, but again there is a marked preference of the enzyme for certain locations of the quinone moiety. Thus, *o*-quinones located at the K-region of PAH such as phenanthrene-9,10-quinone, chrysene- and benz(*a*)-anthracene-5,6-quinone, and benzo(*a*)pyrene-4,5-quinone are among the best substrates of all quinones tested. In addition, *o*-quinones located at the end of an anthracene-type structure such as benz(*a*)anthracene-8,9-quinone, and benzo(*a*)pyrene-7,8- and -9,10-quinone are rapidly reduced by rat liver DDH. A special case is naphthalene-1,2-quinone which non-enzymatically oxidizes NADPH at a rapid rate; this rate is further increased upon addition of rat liver DDH, and the rate of reaction given in Table

Table 2. Oxidation of alcohols by purified rat liver DDH

Substrate	Concentration	Relative reaction rate (%)
Benzene dihydrodiol/$NADP^+$	10 mM	7.03 U/mg = 100
Benzene dihydrodiol/NAD^+	10 mM	4.6
Ethanol	160 mM	ND
Androstane-3α-ol-17-one	100 μM	47
Etiocholane-3α-ol-17-one	100 μM	115
Cholic acid	100 μM	28

Incubations were performed in 50 mM Tris–HCl buffer, pH 9.0, in the presence of 2.3 mM of cofactor.
ND, not detectable.

Table 3. Reduction of aldehydes and ketones by purified rat liver DDH

Substrate	Concentration (mM)	Relative reaction rate (%)
p-Nitrobenzaldehyde	1	2.88 U/mg = 100
p-Nitroacetophenone	1	56
Glucuronic acid	40	0.5
Xylose	40	ND
Glyceraldehyde	4	3.4

Incubations were performed in 50 mM sodium phosphate buffer, pH 7.4, in the presence of 100 μM of NADPH.
ND, not detectable.

4 (179%) is the difference in the reaction rate in the presence and absence of enzyme. Finally, the data in Table 4 give evidence that a methyl substituent adjacent to the quinonoid carbonyl group to be reduced strongly impairs the enzymatic reaction; this is apparent from the reaction rates of duroquinone compared with 1,4-benzoquinone as well as menadione compared with naphthalene-1,4-quinone.

Oxygen consumption during quinone reduction

During the reduction of quinones it was repeatedly observed that small amounts of quinones (i.e. 10 μM benz(*a*)anthracene-5,6-quinone) in the incubation mixture led to the oxidation of rather large amounts of NADPH (>30 μM). This observation suggested the presence of a redox-cycling process, i.e. the quinone which was enzymatically reduced to a hydroquinone may be reoxidized under aerobic conditions by molecular oxygen which is reduced to superoxide anion or hydrogen peroxide. Re-oxidized quinone can then again lead to NADPH reduction, causing a depletion of NADPH and the formation of reactive oxygen species [30, 31]. In order to test this hypothesis, we measured the concentration of oxygen during quinone reduction using a Clark-type oxygen electrode. The results shown in Table 5 demonstrate that oxygen was indeed consumed during the reduction of both K-region and non-K-region *o*-quinones in amounts which were 25–60% of the NADPH oxidized under identical conditions. In contrast, the reduction of 1,4-benzoquinone was not accompanied by oxygen consumption and redox cycling. Preliminary experiments (not shown) indicate that the amount of oxygen consumed during quinone reduction was attenuated after the addition of superoxide dismutase and catalase indicating a role for superoxide anion and hydrogen peroxide in the redox-cycling process.

DISCUSSION

DDH is an interesting enzyme in several respects. The enzyme catalyses the oxidation of a variety of aromatic dihydrodiol metabolites including carcinogenic M-region dihydrodiols of chrysene, (methylated) benz(*a*)anthracene and possibly benzo(*a*)pyrene [4–8]. The oxidation of these dihydrodiols to catechols prevents the enzymatic formation of the highly carcinogenic dihydrodiol epoxides. In the case of benzene and naphthalene, however, the polyhydroxylated metabolites are considered as being the predominant toxic species. Hence, DDH is involved in enzymatic processes of toxification and detoxification in the metabolism of aromatic structures. In rat liver, the DDH activity is associated with the major form of 3α-HSDH in this organ. Several efforts to purify and characterize DDH/3α-HSDH from rat liver have been reported [2, 3, 26, 27, 32]. Moreover, DDH/3α-HSDH is involved in the metabolism and transport of bile acids in hepatocytes [33, 34]. Finally, the enzyme is capable of reducing various hydroxyprostaglandins and may be a target enzyme of anti-inflammatory drugs [35, 36]. Rat liver DDH has recently been closed, and the nucleotide sequence indicates a close relationship to prostaglandin F synthetase and other members of the aldehyde/aldose reductase family [37, 38]. In the present study, we describe a newly developed purification scheme which allows the preparation of a pure and highly active enzyme in 46% yield. It is anticipated that this simple procedure will stimulate further research on this important enzyme.

Topology of the enzyme active site

The major goal of the present study was to investigate the ability of rat liver DDH in quinone

Table 4. Reduction of quinones by purified rat liver DDH

Substrate	Concentration (μM)	Relative reaction rate (%)
p-Benzoquinone	100	8.15 U/mg = 100
Duroquinone	100	10
Naphthalene-1,2-quinone	100	179
Naphthalene-1,4-quinone	100	25
Menadione	100	1.2
Phenanthrene-1,2-quinone	25	ND
Phenanthrene-3,4-quinone	25	1
Phenanthrene-9,10-quinone	25	111
Phenanthrene-1,4-quinone	25	ND
Anthracene-9,10-quinone	25	ND
Benz(*a*)anthracene-1,2-quinone	10	2.6
Benz(*a*)anthracene-3,4-quinone	10	3.5
Benz(*a*)anthracene-5,6-quinone	10	76
Benz(*a*)anthracene-8,9-quinone	10	138
Chrysene-3,4-quinone	10	10
Chrysene-5,6-quinone	10	24
Pyrene-1,6-quinone	10	ND
Pyrene-1,8-quinone	10	ND
Benzo(*a*)pyrene-4,5-quinone	10	39
Benzo(*a*)pyrene-7,8-quinone	10	20
Benzo(*a*)pyrene-9,10-quinone	10	8.6
Benzo(*a*)pyrene-1,6-quinone	10	ND
Benzo(*a*)pyrene-3,6-quinone	10	6.0
Benzo(*a*)pyrene-6,12-quinone	10	1.7

Incubations were performed in 50 mM sodium phosphate buffer, pH 7.4, in the presence of 100 μM of NADPH.
ND, not detectable.

reduction. We found that, in the presence of reduced coenzyme, DDH can act as a quinone reductase whereby the rates of reaction for quinone reduction are often higher at physiological pH than the rates for dihydrodiol oxidation. Moreover, the substrate specificity of rat liver DDH suggests that the binding site for quinones is quite different from the one postulated from our previous data on dihydrodiol oxidation [8]. It is evident that the binding of the reduced coenzyme. NADPH, to the enzyme induces a shift in the conformation of the enzyme active site thereby changing the substrate specificity of the enzyme for quinonoid compounds. A possible visualization of the quinone binding site is schematically depicted in Fig. 3. The site of reduction is located at the upper right and the ketone group to be reduced is marked with an arrow. The major binding region is shown as an angular pocket which directs the quinone group to a relatively hydrophilic binding site where it may come into contact with the cofactor. The angular geometry must be assumed in order to explain the efficient reduction of K-region quinones. It may be noted that no evidence for an angular binding region was obtained in our earlier

Table 5. Oxidation of NADPH and consumption of molecular oxygen during quinone redox cycling catalysed by rat liver DDH

Substrate	NADPH oxidation (μmol/min·mg)	Oxygen consumption (μmol/min·mg)
1,4-Benzoquinone	8.15	ND
Phenanthrene-9,10-quinone	9.05	2.35
Benz(*a*)anthracene-5,6-quinone	6.19	3.59
Benz(*a*)anthracene-8,9-quinone	11.25	3.47

Incubations were performed in 50 mM sodium phosphate buffer, pH 7.4, in the presence of 100 μM of NADPH. NADPH oxidation was measured by the change of absorption at 340 nm in a photometer, and oxygen consumption was determined using a Clark-type electrode.
ND, not detectable.

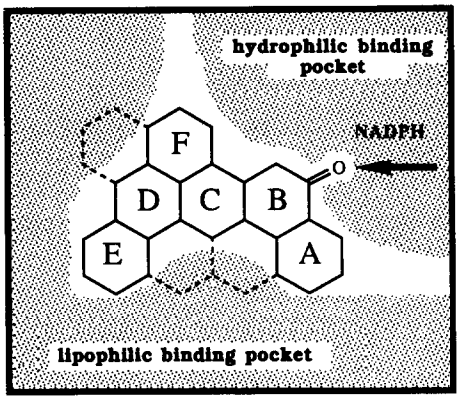

Fig. 3. Hypothetical model of the active site of rat liver DDH as suggested from the results of the present study. Quinonoid substrates which are oriented towards the enzyme active site (arrow marks site of reduction) are accepted as substrates if the aromatic backbone fits into the indicated lipophilic substrate binding pocket (e.g. benz(*a*)anthracene-8,9-quinone is oriented towards the arrow if its ring system occupies the positions BCDE).

study in which the regiospecific oxidation of polycyclic dihydrodiols was investigated [8]. A second ketone group adjacent to the site of reduction is apparently tolerated since many *o*-quinones are good substrates of the enzyme. A methyl substitution, however, such as that found in the poor substrates menadione and duroquinone, seems to disturb substrate binding. Similarly, the mono- or bis-adducts of glutathion with 1,4-benzoquinone and naphthalene-1,4-quinone have been found to be very poor substrates of rat liver DDH (K. Post and M. Przybylski, unpublished). In contrast to the relatively rigid exclusion of substitutions in the immediate vicinity of the enzyme active site, a large hydrophobic pocket must be hypothesized close to the active site which allows the interaction with bulky polycyclic substrates such as benzo(*a*)pyrene-4,5-quinone. The hypothetical scheme depicted in Fig. 3 allows the correct binding of the good substrates, e.g. naphthalene-1,2- and 1,4-quinone (aromatic rings A and B), phenanthrene-9,10-quinone (A,B,C), benz(*a*)anthracene-5,6-quinone (A,B,C,D) and -8,9-quinone (B,C,D,E), and chrysene-5,6-quinone (A,B,C,F). It hinders the binding of the poor substrates, e.g. phenanthrene-1,2- and -1,4-quinone, anthracene-9,10-quinone, and benz(*a*)anthracene-1,2-quinone and therefore seems to be largely compatible with the results of the substrate specificity study compiled in Table 4.

Toxicological significance of quinone reduction

Quinones are prominent products of the biotransformation of aromatic compounds. Naphthalene-1,2-quinone is a metabolite of naphthalene which has been implicated as the species responsible for the oculotoxicity of naphthalene [39, 40]. Naphthalene-1,2-quinone is in fact formed by the oxidation of naphthalene-1,2-dihydrodiol by ocular DDH [41] followed by spontaneous oxidation of the catechol yielding the *o*-quinone. The formation of large amounts of quinones (for naphthalene-1,2-quinone, cf. Ref. 42) is potentially toxic due to the chemical potential of quinones for protein binding, gluthathione depletion and the initiation of redox cycling [30, 31]. As reported above, enzymatic reduction by DDH accelerates the rate of NADPH consumption by naphthalene-1,2-quinone and may therefore contribute to the toxicity of this compound.

1,4-Benzoquinone, as an oxidation product of hydroquinone, is one of the reactive benzene metabolites causing bone marrow toxicity [43, 44]. The reduction of this quinone by DDH can be interpreted as a detoxication process since the hydroquinone formed can be conjugated with sulfate or glucuronate and excreted. The presence of redox cycling during the reduction of 1,4-benzoquinone is unlikely since no oxygen was consumed during this process (Table 5).

Furthermore, in the present study, rat liver DDH has been found to reduce M-region *o*-quinones to the corresponding catechols at rates which are considerably faster than the rates of oxidation of the M-region dihydrodiols by the same enzyme, in particular at physiological pH values. Thus, at a substrate concentration of 10 μM, the oxidation of benz(*a*)anthracene-3,4-dihydrodiol by rat liver DDH proceeds at a rate of 5.5 nmol/min · mg (see Ref. 6)

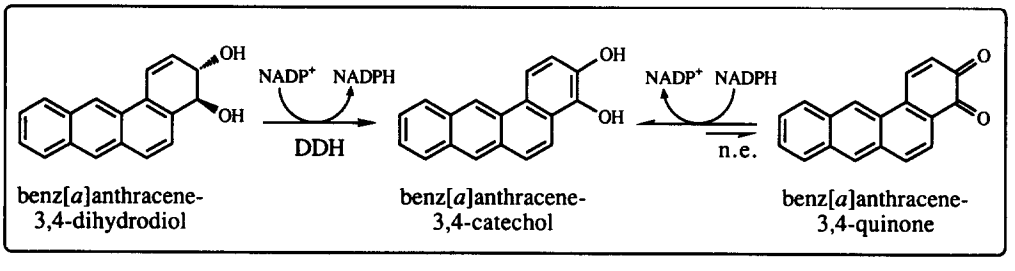

Fig. 4. Formation of 3,4-dihydroxybenz(*a*)anthracene by rat liver DDH. Catechols are formed by rat liver DDH either via enzymatic oxidation of the corresponding dihydrodiol or via enzymatic reduction from the corresponding *o*-quinone. The formation of catechol is favoured because the enzymatic oxidation of dihydrodiol is irreversible, and because the NADPH/$NADP^+$ ratio is high in the cytoplasm which leads to a rapid reduction of quinone. n.e., non-enzymatic reaction.

compared with a rate of 285 nmol/min · mg calculated for the reduction of the benz(*a*)anthracene-3,4-quinone (Fig. 4). Considering the high NADPH/$NADP^+$ ratio in the vital hepatocyte [45], the enzymatic activity of rat liver DDH would be expected to keep the M-region metabolites at the oxidation level of the catechol which may be easily conjugated and excreted and thus to limit the formation of the potentially toxic quinones (Fig. 4). This argument underlines the detoxifying role of DDH in the metabolism of carcinogenic M-region PAH dihydrodiols.

PAH quinones such as benzo(*a*)pyrene-1,6-, -3,6- and -6,12-quinone can be reduced by DT-diaphorase and subsequently conjugated to glucuronic acid [46, 47]. The present study reveals poor activities of rat liver DDH towards these substrates, but rapid reaction rates with *o*-quinones located in the K-region. Rapid reductions of K-region quinones have also been reported as a feature of carbonyl reductases of human liver [48] and placenta [49, 50], and the formation of superoxide anion and hydrogen peroxide has been noted in the latter study [50]. In the present study, we report that the enzymatic oxidation of NADPH is accompanied by the consumption of molecular oxygen in the incubation mixture indicating the formation of potentially toxic reactive oxygen species. At present, it is difficult to estimate the toxicological significance of these findings, since K-region quinones have not been found as major products in microsomal metabolism studies of PAH. It must be noted, however, that phenanthrene-9,10-quinone has been identified as a urinary metabolite of phenanthrene in the rabbit [51, 52] and that it is highly mutagenic in the Ames test due to the production of active oxygen species during redox cycling [53]. Furthermore, quinones are easily formed by photooxidation of PAH and may be absorbed as such by inhalation of, for example, diesel exhaust or cigarette tar [54, 55]. If K-region quinones are, therefore, present in the mammalian organism, then the enzymatic reduction of these species by DDH-type activities may be an initiator of potentially toxic secondary reactions leading to the formation of highly reactive oxygen species.

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